(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 November 2001 (29.11.2001)

PCT

(10) International Publication Number
WO 01/90333 A2

(51) International Patent Classification[7]: C12N 9/00

(21) International Application Number: PCT/US01/17118

(22) International Filing Date: 24 May 2001 (24.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/580,515 25 May 2000 (25.05.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/90333 A2

(54) Title: RECOMBINANT BACTERIAL PHYTASES AND USES THEREOF

(57) Abstract: A purified and modified phytase enzyme from *Escherichia coli* K12 appA phytase is provided. The enzyme has phytase activity and improved thermal tolerance as compared with the wild-type enzyme. In addition, the enzyme has improved protease stability at low pH. Glycosylation of the modified phytase provided a further improved enzyme having improved thermal tolerance and protease stability. The enzyme can be produced from native or recombinant host cells and can be used to aid in the digestion of phytase where desired. In particular, the phytase of the present invention can be used in foodstuffs to improve the feeding value of phytase rich ingredients.

RECOMBINANT BACTERIAL PHYTASES AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, the polypeptides of the present invention have been identified as enzymes having phytase activity.

BACKGROUND OF THE INVENTION

Minerals are essential elements for the growth of all organisms. Dietary minerals can be derived from many source materials, including plants. *E.g.*, plant seeds are a rich source of minerals since they contain ions that are complexed with the phosphate groups of phytic acid molecules. These phytate-associated minerals satisfy the dietary needs of some species of farmed organisms, such as multi-stomached ruminants. Accordingly, ruminants do not require dietary supplementation with inorganic phosphate and minerals because microorganisms in the rumen produce enzymes that catalyze conversion of phytate (myo-inositol-hexaphosphate) to inositol and inorganic phosphate. In the process, minerals that have been complexed with phytate are released. The majority of species of farmed organisms, however, are unable to efficiently utilize phytate-associated minerals. Thus, for example, in the livestock production of monogastric animals (*e.g.*, pigs, birds, and fish), feed is commonly supplemented with minerals &/or with antibiotic substances that alter the digestive flora environment of the consuming organism to enhance growth rates.

As such, there are many problematic burdens - related to nutrition, ex vivo processing steps, health and medicine, environmental conservation, and resource management – that are associated with an insufficient hydrolysis of phytate in many applications. The following are non-limiting examples of these problems:

1) The supplementation of diets with inorganic minerals is a costly expense.

2) The presence of unhydrolyzed phytate is undesirable and problematic in many ex vivo applications (*e.g.* by causing the presence of unwanted sludge).

3) The supplementation of diets with antibiotics poses a medical threat to humans and animals alike by increasing the abundance of antibiotic-tolerant pathogens.

4) The discharge of unabsorbed fecal minerals into the environment disrupts and damages the ecosystems of surrounding soils, fish farm waters, and surface waters at large.

5) The valuable nutritional offerings of many potential foodstuffs remain significantly untapped and squandered.

Many potentially nutritious plants, including particularly their seeds, contain appreciable amounts of nutrients, *e.g.* phosphate, that are associated with phytate in a manner such that these nutrients are not freely available upon consumption. The unavailability of these nutrients is overcome by some organisms, including cows and other ruminants, that have a sufficient digestive ability - largely derived from the presence of symbiotic life forms in their digestive tracts – to hydrolyze phytate and liberate the associated nutrients. However, the majority of species of farmed animals, including pigs, fish, chickens, turkeys, as well as other non-ruminant organisms including man, are unable to efficiently liberate these nutrients after ingestion.

Consequently, phytate-containing foodstuffs require supplementation with exogenous nutrients and/or with a source of phytase activity in order to ammend their deficient nutritional offerings upon consumption by a very large number of species of organisms.

In yet another aspect, the presence of unhydrolized phytate leads to problematic consequences in ex vivo processes including - but not limited to - the processing of foodstuffs. In but merely one exemplification, as described in EP0321004-B1 (Vaara *et al.*), there is a step in the processing of corn and sorghum

kernels whereby the hard kernels are steeped in water to soften them. Water-soluble subtances that leach out during this process become part of a corn steep liquor, which is concentrated by evaporation. Unhydrolized phytic acid in the corn steep liquor, largely in the form of calcium and magnesium salts, is associated with phosphorus and deposits an undesirable sludge with proteins and metal ions. This sludge is problematic in the evaporation, transportation and storage of the corn steep liquor. Accordingly, the instantly disclosed phytase molecules - either alone or in combination with other reagents (including but not limited to enzymes, including proteases) - are serviceable not only in this application (*e.g.*, for prevention of the unwanted slugde) but also in other applications where phytate hydrolysis is desirable.

The supplementation of diets with antibiotic substances has many beneficial results in livestock production. For example, in addition to its role as a prophylactic means to ward off disease, the administration of exogenous antibiotics has been shown to increase growth rates by upwards of 3-5%. The mechanism of this action may also involve – in part – an alteration in the digestive flora environment of farmed animals, resulting in a microfloral balance that is more optimal for nutrient absorption.

However, a significant negative effect associated with the overuse of antibiotics is the danger of creating a repository of pathogenic antibiotic-resistant microbial strains. This danger is imminent, and the rise of drug-resistant pathogens in humans has already been linked to the use of antibiotics in livestock. For example, Avoparcin, the antibiotic used in animal feeds, was banned in many places in 1997, and animals are now being given another antibiotic, virginiamycin, which is very similar to the new drug, Synercid, used to replace vancomycin in human beings. However, studies have already shown that some enterococci in farm animals are resistant to Synercid. Consequently, undesired tolerance consequences, such as those already seen with Avoparcin and vancomycin, are likely to reoccur no matter what new antibiotics are used as blanket prophylactics for farmed animals. Accordingly, researchers are calling for tighter controls on drug use in the industry.

The increases in growth rates achieved in animals raised on foodstuffs supplemented with the instantly disclosed phytase molecules matches – if not exceeds – those achieved using antibiotics such as, for example, Avoparcin. Accordingly, the instantly disclosed phytase molecules - either alone or in combination with other reagents (including but not limited to enzymes, including proteases) - are serviceable not only in this application (*e.g.*, for increasing the growth rate of farmed animals) but also in other applications where phytate hydrolysis is desirable.

An environmental consequence is that the consumption of phytate-containing foodstuffs by any organism species that is phytase-deficient - regardless of whether the foodstuffs are supplemented with minerals - leads to fecal pollution resulting from the excretion of unabsorbed minerals. This pollution has a negative impact not only on the immediate habitat but consequently also on the surrounding waters. The environmental alterations occur primarily at the bottom of the food chain, and therefore have the potential to permeate upwards and throughout an ecosystem to effect permanent and catastrophic damage – particularly after years of continual pollution. This problem has the potential to manifest itself in any area where concentrated phytate processing occurs – including *in vivo* (*e.g.* by animals in areas of livestock production, zoological grounds, wildlife refuges, etc.) and *in vitro* (*e.g.* in commercial corn wet milling, ceral steeping processes, etc.) processing steps.

The decision to use exogenously added phytase molecules – whether to fully replace or to augment the use of exogenously administered minerals &/or antibiotics – ultimately needs to pass a test of financial feasibility & cost effectiveness by the user whose livelihood depends on the relevant application, such as livestock production.

Consequently, there is a need for means to achieve efficient and cost effective hydrolysis of phytate in various applications. Particularly, there is a need for means to optimize the hyrolysis of phytate in commercial applications. In a particular aspect, there is a need to optimize commercial treatment methods that improve the nutritional offerings of phytate-containing foodstuffs for consumption by humans and farmed animals.

Previous reports of recombinant phytases are available, but their inferior activities are eclipsed by the newly discovered phytase molecules of instant invention. Accordingly, the instantly disclosed phytase molecules are counted upon to provide substantially superior commercial performance than previously identified phytase molecules, *e.g.* phytase molecules of fungal origin.

Phytate occurs as a source of stored phosphorous in virtually all plant feeds (Graf (Ed.), 1986). Phytic acid forms a normal part of the seed in cereals and legumes. It functions to bind dietary minerals that are essential to the new plant as it emerges from the seed. When the phosphate groups of phytic acid are removed by the seed enzyme phytase, the ability to bind metal ions is lost and the minerals become available to the plant. In livestock feed grains, the trace minerals bound by phytic acid are largely unavailable for absorption by monogastric animals, which lack phytase activity.

Although some hydrolysis of phytate occurs in the colon, most phytate passes through the gastrointestinal tract of monogastric animals and is excreted in the manure contributing to fecal phosphate pollution problems in areas of intense livestock production. Inorganic phosphorous released in the colon has an appreciably diminished nutritional value to livestock because inorganic phosphorous is absorbed mostly – if not virtually exclusively - in the small intestine. Thus, an appreciable amount of the nutritionally important dietary minerals in phytate is unavailable to monogastric animals.

In sum, phytate-associated nutrients are comprised of not only phosphate that is covalently linked to phytate, but also other minerals that are chelated by phytate as well. Moreover, upon injestion, unhydrolyzed phytate may further encounter and become associated with additional minerals. The chelation of minerals may inhibit the activity of enzymes for which these minerals serve as co-factors.

Conversion of phytate to inositol and inorganic phosphorous can be catalyzed by microbial enzymes referred to broadly as phytases. Phytases such as phytase #EC

3.1.3.8 are capable of catalyzing the hydrolysis of myo-inositol hexaphosphate to D-myo-inositol 1,2,4,5,6-pentaphosphate and orthophosphate. Certain fungal phytases reportedly hydrolyze inositol pentaphosphate to tetra-, tri-, and lower phosphates. *E.g.*, *A. ficuum* phytases reportedly produce mixtures of myoinositol di- and mono-phosphates (Ullah, 1988). Phytase-producing microorganisms are comprised of bacteria such as *Bacillus subtilis* (Powar and Jagannathan, 1982) and Pseudomonas (Cosgrove, 1970); yeasts such as *Sacchoromyces cerevisiae* (Nayini and Markakis, 1984); and fungi such as *Aspergillus terreus* (Yamada *et al.*, 1968).

Acid phosphatases are enzymes that catalytically hydrolyze a wide variety of phosphate esters and usually exhibit pH optima below 6.0 (Igarashi & Hollander, 1968). *E.g.*, #EC 3.1.3.2 enzymes catalyze the hydrolysis of orthophosphoric monoesters to orthophosphate products. An acid phosphatase has reportedly been purified from *A. ficuum*. The deglycosylated form of the acid phosphatase has an apparent molecular weight of 32.6 kDa (Ullah *et al.*, 1987).

Phytase and less specific acid phosphatases are produced by the fungus *Aspergillus ficuum* as extracellular enzymes (Shieh *et al.*, 1969). Ullah reportedly purified a phytase from wild-type *A. ficuum* that had an apparent molecular weight of 61.7 kDA (on SDS-PAGE; as corrected for glycosylation); pH optima at pH 2.5 and pH 5.5; a Km of about 40μm; and, a specific activity of about 50 U/mg (Ullah, 1988). PCT patent application WO 91/05053 also reportedly discloses isolation and molecular cloning of a phytase from *Aspergillus ficuum* with pH optima at pH 2.5 and pH 5.5, a Km of about 250 μm, and specific activity of about 100 U/mg protein.

Summarily, the specific activity cited for these previously reported microbial enzymes has been approximately in the range of 50-100 U/mg protein. In contrast, the phytase activity disclosed in the instant invention has been measured to be approximately 4400 U/mg. This corresponds to about a 40-fold or better improvement in activity.

The possibility of using microbes capable of producing phytase as a feed additive for monogastric animals has been reported previously (USPN 3,297,548 Shieh and Ware; Nelson *et al.*, 1971). The cost-effectiveness of this approach has been a major limitation for this and other commercial applications. Therefore improved phytase molecules are highly desirable.

Microbial phytases may also reportedly be useful for producing animal feed from certain industrial processes, *e.g.*, wheat and corn waste products. In one aspect, the wet milling process of corn produces glutens sold as animal feeds. The addition of phytase may reportedly improve the nutritional value of the feed product. For example, the use of fungal phytase enzymes and process conditions (t~50°C and pH ~5.5) have been reported previously in (*e.g.* EP 0 321 004). Briefly, in processing soybean meal using traditional steeping methods, *i.e.*, methods without the addition of exogenous phytase enzyme, the presence of unhydrolyzed phytate reportedly renders the meal and wastes unsuitable for feeds used in rearing fish, poultry and other non-ruminants as well as calves fed on milk. Phytase is reportedly useful for improving the nutrient and commercial value of this high protein soy material (see Finase Enzymes by Alko, Rajamäki, Finland). A combination of fungal phytase and a pH 2.5 optimum acid phosphatase form *A. niger* has been used by Alko, Ltd as an animal feed supplement in their phytic acid degradative product Finas F and Finase S. However, the cost-effectiveness of this approach has remained a major limitation to more widespread use. Thus a cost-effective source of phytase would greatly enhance the value of soybean meals as an animal feed (Shieh *et al.*, 1969).

To solve the problems disclosed, the treatment of foodstuffs with exogenous phytase enzymes has been proposed, but this approach was not been fully optimized, particularly with respect to feasibility and cost efficiency. This optimization requires the consideration that a wide range of applications exists, particularly for large scale production. For example, there is a wide range of foodstuffs, preparation methods thereof, and species of recipient organisms.

In a particular exemplification, it is appreciated that the manufacture of fish feed pellets requires exposure of ingedients to high temperatures &/or pressure in order to produce pellets that do not dissolve &/or degrade prematurely (*e.g. e.g.* prior to consumption) upon subjection to water. It would thus be desirable for this manufacturing process to obtain additive enzymes that are stable under high temperature and/or pressure conditions. Accordingly it is appreciated that distinct phytases may be differentially preferable or optimal for distinct applications.

It is furthermore recognized that an important way to optimize an enzymatic process is through the modification and improvement of the pivotal catalytic enzyme. For example, a transgenic plant can be formed that is comprised of an expression system for expressing a phytase molecule. It is appreciated that by attempting to improve factors that are not directly related to the activity of the expressed molecule proper, such as the expression level, only a finite - and potentially insufficient - level of optimization may be maximally achieved. Accordingly, there is also a need for obtaining molecules with improved characteristics.

A particular way to achieve improvements in the characteristics of a molecule is through a technological approach termed directed evolution, including Diversa Corporation's proprietary approaches for which the term DirectEvolution® has been coined and registered. These approaches are further elaborated in Diversa's co-owned patent (US 5,830,696) as well as in several co-pending patent applications. In brief, DirectEvolution® comprises: a) the subjection of one or more molecular template to mutagenesis to generate novel molecules, and b) the selection among these progeny species of novel molecules with more desirable characteristics.

However, the power of directed evolution depends on the starting choice of starting templates, as well as on the mutagenesis process(es) chosen and the screening process(es) used. For example, the approach of generating and evaluating a full range of mutagenic permutations on randomly chosen molecular templates &/or on initial molecular templates having overly suboptimal properties is often a forbiddingly large

task. The use of such templates offers, at best, a circuitously suboptimal path and potentially provides very poor prospects of yielding sufficiently improved progeny molecules. Additionally, it is appreciated that our current body of knowledge is very limited with respect to the ability to rigorously predict beneficial modifications.

Consequently, it is a desirable approach to discover and to make use of molecules that have pre-evolved properties – preferably pre-evolved enzymatic advantages – in nature. It is thus appreciated in the instant disclosure that nature provides (through what has sometimes been termed "natural evolution") molecules that can be used immediately in commercial applications, or that alternatively, can be subjected to directed evolution to achieve even greater improvements.

In sum, there is a need for novel, highly active, physiologically effective, and economical sources of phytase activity. Specifically, there is a need to identify novel phytases that: a) have superior activities under one or more specific applications, and are thus serviceable for optimizing these specific applications; b) are serviceable as templates for directed evolution to achieve even further improved novel molecules; and c) are serviceable as tools for the identification of additional related molecules by means such as hybridization-based approaches. This invention meets these needs in a novel way.

SUMMARY OF THE INVENTION

The present invention provides a polynucleotide and a polypeptide encoded thereby which has been identified as a phytase enzyme having phytase activity. In accordance with one aspect of the present invention, there is provided a novel recombinant enzyme, as well as active fragments, analogs and derivatives thereof.

More particularly, this invention relates to the use of recombinant phytase molecules of bacterial origin that are serviceable for improving the nutritional value of phytate-containing foodstuffs. Previous publications have disclosed the use of fungal phytases, but the use of bacterial phyatases for this purpose is novel.

More particularly still, this invention relates to the use of newly identified recombinant phytase molecules of *E.coli* origin that are serviceable for improving the nutritional value of phytate-containing foodstuffs.

This use is comprised of employing the newly identified molecules to hydrolyze phytate in foodstuffs. Hydrolysis may occur before injestion or after injestion or both before and after injestion of the phytate. This application is particularly relevant, but not limited, to non-ruminant organisms and includes the expression of the disclosed novel phytase molecules in transformed hosts, the contacting of the disclosed novel phytase molecules with phytate in foodstuffs and other materials, and the treatment of animal digestive systems with the disclosed novel phytase molecules.

Additionally, hydrolysis may occur independently of consumption, *e.g.* in an *in vitro* application, such as in a reaction vessel. Thus, the treatment of phytate-containing materials includes the treatment of a wide range of materials, including ones that are not intended to be foodstuffs, *e.g.* the treatment of excrementary (or fecal) material.

Preferred molecules of the present invention include a recombinant phytase isolated from *Escherichia coli* B that improves the efficiency of release of phosphorous from phytate and the salts of phytic acid when compared to previosuly identified fungal phytases.

In accordance with one aspect of the present invention, there is provided a phytase enzyme that is serviceable for incorportion into foodstuffs. More specifically, there is provided a phytase enzyme that is serviceable for improving the nutritional value of phytate-containing foodstuffs. More specifically still, there is provided a phytase enzyme that, when applied to phytate-containing foodstuffs, measurably improves the growth performance of an organism that consumes it. It is theorized that the beneficial mechanism of action of the phytase activity is comprised appreciably if not substantially of the hydrolysis of phytate. It is provided that the beneficial action

may occur before injestion or alternatively after injestion or alternatively both before and after injestion of the phytate-containing foodstuff. In the case where the beneficial action occurs after injestion, it is an object of the present invention to provide a phytase enzyme that has activity that is retained upon consumption by non-ruminant organisms.

In accordance with another aspect of the present invention there are provided isolated nucleic acid molecules encoding the enzyme of the present invention - including mRNA, DNA, cDNA, genomic DNA - as well as active derivatives, analogs and fragments of such enzyme.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding an enzyme of the present invention, under conditions promoting expression of said enzyme and subsequent recovery of said enzyme.

In accordance with yet a further aspect of the present invention, there is provided a process for expressing such enzymes, or polynucleotides encoding such enzymes in transgenic plants or plant organs and methods for the production of such plants. This is achievable by introducing into a plant an expression construct comprised of a nucleic acid sequence encoding such phytase enzymes.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes for use in commercial processes, such as, for example, processes that liberate minerals from phytates in plant materials either *in vitro*, *i.e.*, in feed treatment processes, or *in vivo*, *i.e.*, by administering the enzymes to animals.

In accordance with yet a further aspect of the present invention, there are provided foodstuffs made by the disclosed feed treatment processes.

In accordance with yet a further aspect of the present invention, there are provided a processes for utilizing such enzymes, or polynucleotides encoding such enzymes, for *in vitro* purposes related to research, discovery, and development. In a non-limiting exemplification, such processes comprise the generation of probes for identifying and isolating similar sequences which might encode similar enzymes from other organisms.

In a particular non-limiting examplification, there are also provided processes for generating nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention. By way of preferred exemplification, hybridization-based uses of these probes include, but are by no means limited to, PCR, Northern and Southern types of hybridizations, RNA protection assays, and in situ types of hybridizations. The uses of the instantly disclosed molecules further include, in a non-limiting manner, diagnostic applications.

In accordance with a non-limiting exemplification, these processes comprise the generation of antibodies to the disclosed molecules, and uses of such antibodies, including, for example, for the identification and isolation of similar sequences in enzymes from other organisms. In another non-limiting examplification, these processes include the use of the present enzymes as templates for directed evolution, comprising the generation of novel molecules by followed by screening-based approaches for discoverying of progeny molecules with improved properties.

Also provided is a transgenic non-human organism whose genome comprises a heterologous nucleic acid sequence encoding a polypeptide having phytase activity, wherein said transgene results in expression of a phytase polypeptide.

The invention also provides phytase encoding polynucleotides having a nucleotide sequence substantially identical to SEQ ID NO:7, and having a modified nucleotide sequence selected from nucleotide 390 is G; 391 is A; nucleotide 438 is T; 439 is G; 440 is G; 471 is C; 473 is T; 477 is T; 448 is G; 449 is T; 690 is G; 691 is A; 692 is G; 729 is T; 730 is A; 731 is T; 864 is T; 865 is G; 1017 is G, or any combination

thereof. Further, the invention provides a polynucleotide having a nucleotide sequence substantially identical to SEQ ID NO:7, and having a modified nucleotide sequence selected from nucleotide 390 is G and 391 is A; nucleotide 438 is T, 439 is G and 440 is G; 471 is C and 473 is T; 477 is T, 448 is G, and 449 is T; 690 is G, 691 is A and 692 is G; 729 is T, 730 is A, and 731 is T; 864 is T and 865 is G; 1017 is G, or any combination thereof. The later sequence is exemplified in SEQ ID NO:9 and the corresponding amino acid sequence is SEQ ID NO:10.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the nucleotide and deduced amino acid sequences the enzyme of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

Figure 2 shows the pH and temperature profile and stability data for the phytase enzyme of the present invention. The assay used for these analysis is the following for the detection of phytase activity: Phytase activity is measured by incubating 150µl of the enzyme preparation with 600µl of 2 mM sodium phytate in 100 mM Tris HCl buffer pH 7.5, supplemented with 1mM $CaCl_2$ for 30 minutes at 37°C. After incubation the reaction is stopped by adding 750µl of 5% trichloroacetic acid. Phosphate released was measured against phosphate standard spectrophotometrically at 700nm after adding 1500µl of the color reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulfuric acid and 1 volume of 2.7% ferrous sulfate; Shimizu, 1992). OD at 700nm is indicated on the Y-axis of the graphs in Figure 2. Temperature or pH is indicated on the X-axis of the graphs.

Figure 3 shows a graph with the results of a thermal tolerance assay between SEQ ID NO:8 and SEQ ID NO:10 (modified phytase).

Figure 4 shows a graph with the stability of phytase enzymes under simulated digestibility conditions.

Figure 5 shows a graph with expression of wild-type and modified phytase (SEQ ID NO:10) in various host cells.

Figure 6 shows a graph of residual phytase activity in SGF with pepsin.

Figure 7 shows the nucleotide sequence of E. coli appA phytase (SEQ ID NO:7).

Figure 8 shows the amino acid sequence of E.coli appA phytase (SEQ ID NO:8) and a modified phytase (SEQ ID NO:10).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a thermally stable phytase having a polynucleotide and polypeptide sequence modified in such a way as to provide a phytase havng increased thermal stability as compared with other phytase enzymes. Expression of this new phytase in *S. pombe* or *P. pastoris*, for example, resulted in the production of glycosylated variants that exhibited additional thermal tolerance.

The present invention provides purified a recombinant phytase enzyme, shown in Figure 1. Additionally, the present invention provides isolated nucleic acid molecules (polynucleotides) which encode for the mature enzyme having the deduced amino acid sequence of Figure 1. The invention also provides modified phytase sequences as shown in Figure 8 and SEQ ID NO:9 and 10.

The phytase molecules of the instant invention are novel with respect to their structures. Additionally, the instant phytase molecules are patentably novel with respect to activity. For example, using an assay (as described in Food Chemicals

Codex, 4th Ed.) the activity of the instant phytase enzyme was demonstrated to be far superior in comparison to a fungal (*Aspergillus*) phytase control. Specifically, a plurality of experiments showed the *E. coli* phytase to have an activity of about 4400 units/mg and the *Aspergillus* phytase to have an activity of about 105 units/mg. This corresponds to more than a 40-fold difference in activity. In order to facilitate understanding of the examples provided herein, certain frequently occurring methods and/or terms will be described.

The term "antibody," as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', $(Fab')_2$, Fv, and SCA fragments, that are capable of binding to an epitope of a phytase polypeptide. These antibody fragments, which retain some ability to selectively bind to the antigen (*e.g.*, an phytase antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, *e.g.*, Harlow and Lane, *supra*), and are described further, as follows.

(1) An Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.

(2) An Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.

(3) An $(Fab')_2$ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A $(Fab')_2$ fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.

(4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

(5) An single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

The term "degrading effective" amount refers to the amount of enzyme which is required to degrade at least 50% of the phytate, as compared to phytate not contacted with the enzyme. Preferably, at least 80% of the phytate is degraded.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a gel to isolate the desired fragment.

As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen, such as a phytase polypeptide, to which the paratope of an antibody, such as an phytase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The terms "fragment", "derivative" and "analog" when referring to the enzyme of Figure 1 comprise a enzyme which retains at least one biological function or activity that is at least essentially same as that of the reference enzyme. Furthermore, the terms "fragment", "derivative" or "analog" are exemplified by a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

By "isolated nucleic acid" is meant a nucleic acid, *e.g.*, a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, *e.g.*, a DNA fragment produced by PCR amplification or restriction enzyme digestion, or an RNA molecule produced by *in vitro* transcription. The term also describes a recombinant

nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook *et al.*, 1989). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

As used herein, a "nucleic acid molecule" is comprised of at least one nucleotide base or one nucleotide base pair, depending on whether it is single-stranded or double-stranded, respectively. Furthermore, a nucleic acid molecule may belong exclusively or chimerically to any group of nucleotide-containing molecules, as exemplified by, but not limited to, the following groups of nucleic acid molecules: RNA, DNA, genomic nucleic acids, non-genomic nucleic acids, naturally occurring and not naturally occurring nucleic acids, and synthetic nucleic acids. This includes, by way of non-limiting example, nucleic acids associated with any organelle, such as the mitochondria, ribosomal RNA, and nucleic acid molecules comprised chimerically of one or more components that are not naturally occurring along with naturally occurring components.

Additionally, a "nucleic acid molecule" may contain in part one or more non-nucleotide-based components as exemplified by, but not limited to, amino acids and sugars. Thus, by way of example, but not limitation, a ribozyme that is in part nucleotide-based and in part protein-based is considered a "nucleic acid molecule".

In addition, by way of example, but not limitation, a nucleic acid molecule that is labeled with a detectable moiety, such as a radioactive or alternatively a non-radioactive label, is likewise considered a "nucleic acid molecule".

The terms "nucleic acid sequence coding for" or a "DNA coding sequence of" or a "nucleotide sequence encoding" a particular enzyme – as well as other

synonymous terms – refer to a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences. A "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background bind. However, after the RNA polymerase binds the sequence and transcription is initiated at the start codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promotor sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The terms "nucleic acid encoding an enzyme (protein)" or "DNA encoding an enzyme (protein)" or "polynucleotide encoding an enzyme (protein)" and other synonymous terms encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

In one preferred embodiment, a "specific nucleic acid molecule species" is defined by its chemical structure, as exemplified by, but not limited to, its primary sequence. In another preferred embodiment, a specific "nucleic acid molecule species" is defined by a function of the nucleic acid species or by a function of a product derived from the nucleic acid species. Thus, by way of non-limiting example, a "specific nucleic acid molecule species" may be defined by one or more activities or properties attributable to it, including activities or properties attributable its expressed product.

The instant definition of "assembling a working nucleic acid sample into a nucleic acid library" includes the process of incorporating a nucleic acid sample into a vector-based collection, such as by ligation into a vector and transformation of a host.

enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

Gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affects high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different phytase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

Therefore, in a one embodiment, the invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;

2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;

3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;

4) screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and

5) isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the art and are discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the invention.

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, bacteriophage 1 insertion vectors or replacement vectors, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes (BAC), viral DNA (*e.g.*, vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes (PAC), yeast plasmids, yeast artificial chromosomes (YAC), and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

A preferred type of vector for use in the present invention contains an f-factor origin replication. The f-factor (or fertility factor) in *E. coli* is a plasmid which effects high frequency transfer of itself during conjugation and less frequent transfer of the bacterial chromosome itself. A particularly preferred embodiment is to use cloning

vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. colt* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library."

Another type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in "Molecular Cloning: A laboratory Manual" (Sambrook *et al.*, 1989).

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include *lacI*, *lacZ*, *T3*, *T7*, *gpt*, *lambda* P_R, P_L and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in *E. coli*.

In vivo reassortment is focused on "inter-molecular" processes collectively referred to as "recombination" which in bacteria, is generally viewed as a "RecA-dependent" phenomenon. The invention can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells' ability to mediate reductive processes to decrease the complexity of quasi-repeated sequences in the cell

by deletion. This process of "reductive reassortment" occurs by an "intra-molecular", RecA-independent process.

Therefore, in another aspect of the invention, variant polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their insertion into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases the efficiency, it may still provide sufficient elasticity for the effective recovery of novel molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNAseH.

b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.

c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced RI. The re-assorted encoding sequences can then be

recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be effected by:

1) The use of vectors only stably maintained when the construct is reduced in complexity.

2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.

3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.

4) The use of direct selection techniques with an expression vector and the appropriate selection.

Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

The following example demonstrates a method of the invention. Encoding nucleic acid sequences (quasi-repeats) derived from three (3) unique species are depicted. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence which are designated "A", "B" and "C". The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly

conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an appropriate host cell. The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. Whether the reduction in RI is mediated by deletion formation between repeated sequences by an "intra-molecular" mechanism, or mediated by recombination-like events through "inter-molecular" mechanisms is immaterial. The end result is a reassortment of the molecules into all possible combinations.

Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact, or catalyze a particular reaction (*e.g.*, such as catalyzing the hydrolysis of a haloalkane).

The polypeptides that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (*e.g.*, catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or selection.

In another aspect, prior to or during recombination or reassortment, polynucleotides of the invention or polynucleotides generated by the method described herein can be subjected to agents or processes which promote the introduction of mutations into the original polynucleotides. The introduction of such mutations would increase the diversity of resulting hybrid polynucleotides and polypeptides encoded therefrom. The agents or processes which promote mutagenesis can include, but are not limited to: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine, see Sun and Hurley, 1992); an N-acelylated or deacetylated 4'-fluro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see, for

example, van de Poll *et al.*, 1992); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see also, van de Poll *et al.*, 1992, pp. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon ("PAH") DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[*a*]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[*a*]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]-quinoline ("N-hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*f*]-pyridine ("N-hydroxy-PhIP"). Especially preferred means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotides pool, which can be released or removed by a process including heating the solution comprising the polynucleotides prior to further processing.

In another aspect the invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under conditions according to the invention which provide for the production of hybrid or re-assorted polynucleotides.

The invention also provides for the use of proprietary codon primers (containing a degenerate N,N,N sequence) to introduce point mutations into a polynucleotide, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position (gene site saturated mutagenesis (GSSM)). The oligos used are comprised contiguously of a first homologous sequence, a degenerate N,N,N sequence, and preferably but not necessarily a second homologous sequence. The downstream progeny translational products from the use of such oligos include all possible amino acid changes at each

amino acid site along the polypeptide, because the degeneracy of the N,N,N sequence includes codons for all 20 amino acids.

In one aspect, one such degenerate oligo (comprised of one degenerate N,N,G/T cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,G/T cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. Thus, more than one N,N,G/T sequence can be contained in one oligo to introduce amino acid mutations at more than one site. This plurality of N,N,G/T sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,G/T sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

In a particular exemplification, it is possible to simultaneously mutagenize two or more contiguous amino acid positions using an oligo that contains contiguous N,N,G/T triplets, *i.e.* a degenerate $(N,N,G/T)_n$ sequence.

In another aspect, the present invention provides for the use of degenerate cassettes having less degeneracy than the N,N,G/T sequence. For example, it may be desirable in some instances to use (*e.g.* in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (*e.g.*, in an oligo) a degenerate N,N,N triplet sequence, or an N,N, G/C triplet sequence.

It is appreciated, however, that the use of a degenerate triplet (such as N,N,G/T or an N,N, G/C triplet sequence) as disclosed in the instant invention is advantageous for several reasons. In one aspect, this invention provides a means to systematically

and fairly easily generate the substitution of the full range of possible amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide. Thus, for a 100 amino acid polypeptide, the invention provides a way to systematically and fairly easily generate 2000 distinct species (*i.e.*, 20 possible amino acids per position times 100 amino acid positions). It is appreciated that there is provided, through the use of an oligo containing a degenerate N,N,G/T or an N,N, G/C triplet sequence, 32 individual sequences that code for 20 possible amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using one such oligo, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligo in site-directed mutagenesis leads to only one progeny polypeptide product per reaction vessel.

This invention also provides for the use of nondegenerate oligos, which can optionally be used in combination with degenerate primers disclosed. It is appreciated that in some situations, it is advantageous to use nondegenerate oligos to generate specific point mutations in a working polynucleotide. This provides a means to generate specific silent point mutations, point mutations leading to corresponding amino acid changes, and point mutations that cause the generation of stop codons and the corresponding expression of polypeptide fragments.

Thus, in one embodiment, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide molecules such that all 20 amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide. The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel can be subjected to clonal amplification (*e.g.*, cloned into a suitable *E. coli* host using an expression vector) and subjected to expression screening. When an individual progeny polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide), it can be sequenced to identify the correspondingly favorable amino acid substitution contained therein.

It is appreciated that upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid changes may be identified at more than one amino acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if 2 specific favorable amino acid changes are identified in each of 3 amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes) and 3 positions. Thus, there are 3 x 3 x 3 or 27 total possibilities, including 7 that were previously examined - 6 single point mutations (*i.e.*, 2 at each of three positions) and no change at any position.

In yet another aspect, site-saturation mutagenesis can be used together with shuffling, chimerization, recombination and other mutagenizing processes, along with screening. This invention provides for the use of any mutagenizing process(es), including saturation mutagenesis, in an iterative manner. In one exemplification, the iterative use of any mutagenizing process(es) is used in combination with screening.

Thus, in a non-limiting exemplification, polynucleotides and polypeptides of the invention can be derived by saturation mutagenesis in combination with additional mutagenization processes, such as process where two or more related polynucleotides are introduced into a suitable host cell such that a hybrid polynucleotide is generated by recombination and reductive reassortment.

In addition to performing mutagenesis along the entire sequence of a gene, mutagenesis can be used to replace each of any number of bases in a polynucleotide sequence, wherein the number of bases to be mutagenized is preferably every integer from 15 to 100,000. Thus, instead of mutagenizing every position along a molecule, one can subject every or a discrete number of bases (preferably a subset totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer, containing a heterologous cassette, also referred

to as a mutagenic cassette. Preferred cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, C/G/T, A/G/T, A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo).

In a general sense, saturation mutagenesis is comprised of mutagenizing a complete set of mutagenic cassettes (wherein each cassette is preferably about 1-500 bases in length) in defined polynucleotide sequence to be mutagenized (wherein the sequence to be mutagenized is preferably from about 15 to 100,000 bases in length). Thus, a group of mutations (ranging from 1 to 100 mutations) is introduced into each cassette to be mutagenized. A grouping of mutations to be introduced into one cassette can be different or the same from a second grouping of mutations to be introduced into a second cassette during the application of one round of saturation mutagenesis. Such groupings are exemplified by deletions, additions, groupings of particular codons, and groupings of particular nucleotide cassettes.

Defined sequences to be mutagenized include a whole gene, pathway, cDNA, an entire open reading frame (ORF), and entire promoter, enhancer, repressor/transactivator, origin of replication, intron, operator, or any polynucleotide functional group. Generally, a "defined sequences" for this purpose may be any polynucleotide that a 15 base-polynucleotide sequence, and polynucleotide sequences of lengths between 15 bases and 15,000 bases (this invention specifically names every integer in between). Considerations in choosing groupings of codons include types of amino acids encoded by a degenerate mutagenic cassette.

In a particularly preferred exemplification a grouping of mutations that can be introduced into a mutagenic cassette, this invention specifically provides for degenerate codon substitutions (using degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 amino acids at each position, and a library of polypeptides encoded thereby.

The present invention also includes polynucleotides, wherein the coding sequence for the mature enzyme may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of an enzyme from a host cell, for example, a leader sequence which functions to control transport of an enzyme from the cell. An enzyme having a leader sequence is an example of a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the enzyme. The polynucleotides may also encode for a proprotein which is exemplified by a mature protein plus additional 5' amino acid residues. An otherwise mature protein having a prosequence is exemplified by a proprotein that is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature enzyme, or for an enzyme having a prosequence or for an enzyme having both a prosequence and a presequence (*e.g.* leader sequence).

The coding sequences for the phytase enzymes of the present invention were identified by preparing *E.coli* B genomic DNA, for example, and recovering (via, for example, PCR amplification) from the genomic DNA, DNA encoding phytase activity. Such methods for recovery are well-known in the art. One means, for example, comprises designing amplification primers to recover the coding sequence, amplifying the gene from the genomic DNA, subcloning the DNA into a vector, transforming the resulting construct into a host strain, and expressing the phytase enzyme for evaluation. Such procedures are well known in the art and methods are provided, for example, in Sambrook *et al.*, 1989, which is hereby incorporated by reference in its entirety.

In a preferred embodiment, the enzyme of the present invention, was isolated from an *E.coli* B genomic DNA by the following technique:

E.coli B genomic DNA was obtained comercially (Sigma: Catalog # D-2001, St. Louis, New Jersey).The following primers were used to amplify the gene directly from the genomic DNA:

5' primer gtttctgaattcaaggaggaatttaaATGAAAGCGATCTTAATCCCATT (SEQ ID NO:3); and

3' primer gtttctggatccTTACAAACTGCACGCCGGTAT (SEQ ID NO:4)

Pfu polymerase was used according to manufacturers protocol (Stratagene Cloning Systems, Inc., La Jolla, CA).

PCR product and pQE60 vector (Qiagen) were both digested with EcoRI and BglII restriction endonucleases (New England Biolabs) according to manufacturers protocols. Ligation and transformation into, and expression in M15 pREP4 host cells (Qiagen) yields c-term 6X-His tagged protein.

The isolated nucleic acid sequences and other enzymes may then be measured for retention of biological activity characteristic to the enzyme of the present invention, for example, in an assay for detecting enzymatic phytase activity (Food Chemicals Codex, 4th Ed.). Such enzymes include truncated forms of phytase, and variants such as deletion and insertion variants.

An *in vitro* example of such an assay is the following assay for the detection of phytase activity: Phytase activity can be measured by incubating 150µl of the enzyme preparation with 600µl of 2 mM sodium phytate in 100 mM Tris HCl buffer pH 7.5, supplemented with 1mM $CaCl_2$ for 30 minutes at 37°C. After incubation the reaction is stopped by adding 750µl of 5% trichloroacetic acid. Phosphate released was measured against phosphate standard spectrophotometrically at 700nm after adding 1500µl of the color reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulfuric acid and 1 volume of 2.7% ferrous sulfate; Shimizu, 1992). One unit of enzyme activity is defined as the amount of enzyme required to liberate one µmol Pi

per min under assay conditions. Specific activity can be expressed in units of enzyme activity per mg of protein.

The enzyme of the present invention has enzymatic activity with respect to the hydrolysis of phytate to inositol and free phosphate.

The enzymes and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity. The phytase polypeptide of the invention can be obtained using any of several standard methods. For example, phytase polypeptides can be produced in a standard recombinant expression system (see below), chemically synthesized (this approach may be limited to small phytase peptide fragments), or purified from organisms in which they are naturally expressed. Serviceable recombinant expression methods include the use of mammalian hosts, microbial hosts, and plant hosts.

The recombinant expression of the instant phytase molecules may be achieved in combination with one or more additional molecules such as, for example, other enzymes. This approach is serviceable for producing combination products, such as a plant or plant part that contains the instant phytase molecules as well as one or more additional molecules – preferably said phytase molecules and said additional molecules are serviceable in a combination treatment. The resulting recombinantly expresssed molecules may be used in homogenized and/or purified form or alternatively in relatively unpurified form (*e.g.* as consumable plant parts that are serviceable when admixed with other foodstuffs for catalyzing the degredation of phytate).

In sum, in a non-limiting embodiment, the present invention provides a recombinant enzyme expressed in a host. In another non-limiting embodiment, the present invention provides a substantially pure phytase enzyme. Thus, an enzyme of the present invention may be a recombinant enzyme, a natural enzyme, or a synthetic enzyme, preferably a recombinant enzyme.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention, and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (*e.g.* transduced or transformed or transfected) with the vectors containing the polynucleotides of this invention. Such vectors may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, a prion, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, &/or selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Inclusive in this meaning is the use of blunt-ended molecules which could be generated by the use of restriction digestion as well as restriction digestion-independent means. Alternatively, the insert may be incorporated into a vector by so called "ligase-independent" means. In a particular aspect, a "ligase-independent" means is exemplified by the use of topoisomerase-mediated ligation at room temperature, for example according to the commercially available kit termed TOPO-TA Cloning® (Invitrogen Corporation,

Carlsbad, CA). Alteranative enzymes, including isomers of topoisomerase as well as more distantly related recombination enzymes (*e.g.* recombinases), may also be serviceable for mediating this type of "ligase-independent" incorporation. In another particular aspect, a "ligase-independent" means is exemplified by the use host repair mechanisms. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: an LTR or SV40 promoter, an *E. coli. lac* or *trp*, a phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli.*

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, Streptomyces, *Bacillus subtilis*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, *etc.* The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. One or more additional inserts may also be incorporated that lead to expression of one or more aditional molecules, such as another phytase or a protease enzyme, preferably said one or more additional molecules are serviceable in combination with the instant phytase in a combination treatment.

Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. "Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II (Stratagene); pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmids or other vectors may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, 1986).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzymes of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described (*e.g.* Sambrook *et al.*, 1989, the disclosure of which is hereby incorporated by reference).

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), Å-factor, acid phosphatase, or heat shock proteins,

among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis, Salmonella typhimurium* and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period.

substrate or the enzyme or both. It is additionally appreciated that the phytate substrate may be contacted with – in addition to the phytase – one or more additional reagents, such as another enzyme, which may be also be applied either directly or after purification from its source material.

It is appreciated that the phytase source material(s) can be contacted directly with the phytate source material(s); *e.g.* upon *in vitro* or *in vivo* grinding or chewing of either or both the phytase source(s) and the phytate source(s). Alternatively the phytase enzyme may be purified away from source material(s), or the phytate substrate may be purified away from source material(s), or both the phytase enzyme and the phytate substrate may be purified away from source material(s) prior to the contacing of the phytase enzyme with the phytate substrate. It is appreciated that a combination of purified and unpurified reagents - including enzyme(s) or substrates(s) or both – may be used.

It is appreciated that more than one source material may be used as a source of phytase activity. This is serviceable as one way to achieve a timed release of reagent(s) from source material(s), where release from different reagents from their source materials occur differentially, for example as injested source materials are digested *in vivo* or as source materials are processed in *in vitro* applications. The use of more than one source material of phytase activity is also serviceable to obtain phytase activities under a range of conditions and fluctuations thereof, that may be encountered - such as a range of pH values, temperatures, salinities, and time intervals – for example during different processing steps of an application. The use of different source materials is also serviceable in order to obtain different reagents, as exemplified by one or more forms or isomers of phytase and/or phytate &/or other materials.

It is appreciated that a single source material, such a trangenic plant species (or plant parts thereof), may be a source material of both phytase and phytate; and that enzymes and substrates may be differentially compartmentalized within said single source – *e.g.* secreted vs. non-secreted, differentially expressed &/or having

differential abundances in different plant parts or organs or tissues or in subcellular compartments within the same plant part or organ or tissue. Purification of the phytase molecules contained therein may comprise isolating and/or further processing of one or more desirable plant parts or organs or tissues or subcellular compartments.

In a particular aspect, this invention provides a method of catalyzing *in vivo* and/or *in vitro* reactions using seeds containing enhanced amounts of enzymes. The method comprises adding transgenic, non-wild type seeds, preferably in a ground form, to a reaction mixture and allowing the enzymes in the seeds to increase the rate of reaction. By directly adding the seeds to the reaction mixture the method provides a solution to the more expensive and cumbersome process of extracting and purifying the enzyme. Methods of treatment are also provided whereby an organism lacking a sufficient supply of an enzyme is administered the enzyme in the form of seeds from one or more plant species, preferably transgenic plant species, containing enhanced amounts of the enzyme. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,543,576 (Van Ooijen *et al.*) and USPN 5,714,474 (Van Ooijen *et al.*), although these reference do not teach the inventive molecules of the instant application and instead teach the use of fungal phytases.

In a particular non-limiting aspect, the instant phytase molecules are serviceable for generating recombinant digestive system life forms (or microbes or flora) and for the administration of said recombinant digestive system life forms to animals. Administration may be optionally performed alone or in combination with other enzymes &/or with other life forms that can provide enzymatic activity in a digestive system, where said other enzymes and said life forms may be may recombinant or otherwise. For example, administration may be performed in combination with xylanolytic bacteria

In a non-limiting aspect, the present invention provides a method for steeping corn or sorghum kernels in warm water containing sulfur dioxide in the presence of an

enzyme preparation comprising one or more phytin-degrading enzymes, preferably in such an amount that the phytin present in the corn or sorghum is substantially degraded. The enzyme preparation may comprise phytase and/or acid phosphatase and optionally other plant material degrading enzymes. The steeping time may be 12 to 18 hours. The steeping may be interrupted by an intermediate milling step, reducing the steeping time. In a preferred embodiment, corn or sorghum kernels are steeped in warm water containing sulfur dioxide in the presence of an enzyme preparation including one or more phytin-degrading enzymes, such as phytase and acid phosphatases, to eliminate or greatly reduce phytic acid and the salts of phytic acid. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 4,914,029 (Caransa *et al.*) and EP 0321004 (Vaara *et al.*), although these reference do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method to obtain a bread dough having desirable physical properties such as non-tackiness and elasticity and a bread product of superior quality such as a specific volume comprising adding phytase molecules to the bread dough. In a preferred embodiment, phytase molecules of the instant invention are added to a working bread dough preparation that is subsequently formed and baked. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 03076529 (Hara *et al.*), although this reference does not teach the inventive phytase molecules of the instant application.

In a non-limiting aspect, the present invention provides a method to produce improved soybean foodstuffs. Soybeans are combined with phytase molecules of the instant invention to remove phytic acid from the soybeans, thus producing soybean foodstuffs that are improved in their supply of trace nutrients essential for consuming organisms and in its digestibility of proteins. In a preferred embodiment, in the

production of soybean milk, phytase molecules of the instant invention are added to or brought into contact with soybeans in order to reduce the phytic acid content. In a non-limiting exemplification, the application process can be accelerated by agitating the soybean milk together with the enzyme under heating or by a conducting a mixing-type reaction in an agitation container using an immobilized enzyme. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 59166049 (Kamikubo *et al.*), although this reference does not teach the inventive molecules of the instant application.

In one aspect, the instant invention provides a method of producing an admixture product for drinking water or animal feed in fluid form, and which comprises using mineral mixtures and vitamin mixtures, and also novel phytase molecules of the instant invention. In a preferred embodiment, there is achieved a correctly dosed and composed mixture of necessary nutrients for the consuming organism without any risk of precipitation and destruction of important minerals/vitamins, while at the same time optimum utilization is made of the phytin-bound phosphate in the feed. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes EP 0772978 (Bendixen *et al.*), although this reference does not teach the inventive molecules of the instant application.

It is appreciated that the phytase molecules of the instant invention may also be used to produce other alcoholic and non-alcoholic drinkable foodstuffs (or drinks) based on the use of molds &/or on grains &/or on other plants. These drinkable foodstuffs include liquors, wines, mixed alcoholic drinks (*e.g.* wine coolers, other alcoholic coffees such as Irish coffees, etc.), beers, near-beers, juices, extracts, homogenates, and purees. In a preferred exemplification, the instantly disclosed phytase molecules are used to generate transgenic versions of molds &/or grains &/or other plants serviceable for the production of such drinkable foodstuffs. In another

preferred exemplification, the instantly disclosed phytase molecules are used as additional ingredients in the manufacturing process &/or in the final content of such drinkable foodstuffs. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. However - due to the novelty of the instant invention - references in the publicly available literature do not teach the inventive molecules instantly disclosed.

In another non-limiting exemplification, the present invention provides a means to obtain refined sake having a reduced amount of phytin and an increased content of inositol. Such a sake may have – through direct &/or psychogenic effects - a preventive action on hepatic disease, arteriosclerosis, and other diseases. In a preferred embodiment, a sake is produced from rice Koji by multiplying a rice Koji mold having high phytase activity as a raw material. It is appreciated that the phytase molecules of the instant invention may be used to produce a serviceable mold with enhanced activity (preferably a transgenic mold) &/or added exogenously to augment the effects of a Koji mold. The strain is added to boiled rice and Koji is produced by a conventional procedure. In a preferred exemplification, the prepared Koji is used, the whole rice is prepared at two stages and Sake is produced at constant Sake temperature of 15°C to give the objective refined Sake having a reduced amount of phytin and an increased amount of inositol. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 06153896 (Soga *et al.*) and JP 06070749 (Soga *et al.*), although these references do not teach the inventive molecules of the instant application

In a non-limiting aspect, the present invention provides a method to obtain an absorbefacient capable of promoting the absorption of minerals including ingested calcium without being digested by gastric juices or intestinal juices at a low cost. In a preferred embodiment, said mineral absorbefacient contains a partial hydrolysate of phytic acid as an active ingredient. Preferably, a partial hydrolyzate of the phytic acid is produced by hydrolyzing the phytic acid or its salts using novel phytase molecules

of the instant invention. The treatment with said phytase molecules may occur either alone &/or in a combination treatment (to inhibit or to augment the final effect), and is followed by inhibiting the hydrolysis within a range so as not to liberate all the phosphate radicals. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 04270296 (Hoshino), although reference in the publicly available literature do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method (and products therefrom) to produce an enzyme composition having an additive or preferably a synergistic phytate hydrolyzing activity; said composition comprises novel phytase molecules of the instant invention and one or more additional reagents to achieve a composition that is serviceable for a combination treatment. In a preferred embodiment, the combination treatment of the present invention is achieved with the use of at least two phytases of different position specificity, *i.e.* any combinations of 1-, 2-, 3-, 4-, 5-, and 6-phytases. By combining phytases of different position specificity an additive or synergistic effect is obtained. Compositions such as food and feed or food and feed additives comprising such phytases in combination are also included in this invention as are processes for their preparation. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes WO9 830681 (Ohmann *et al.*), although references in the publicly available literature do not teach the use of the inventive molecules of the instant application.

In another preferred embodiment, the combination treatment of the present invention is achieved with the use of an acid phosphatase having phytate hydrolyzing activity at a pH of 2.5, in a low ratio corresponding to a pH 2.5:5.0 activity profile of from about 0.1:1.0 to 10:1, preferably of from about 0.5:1.0 to 5:1, more preferably still of from about 0.8:1.0 to 3:1, and more preferably still of from about 0.8:1.0 to

2:1. Said enzyme composition preferably displays a higher synergetic phytate hydrolyzing efficiency through thermal treatment. Said enzyme composition is serviceable in the treatment of foodstuffs (drinkable and solid food, feed and fodder products) to improve phytate hydrolysis. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,554,399 (Vanderbeke *et al.*) and USPN 5,443,979 (Vanderbeke *et al.*), although these reference do not teach the use of the inventive molecules of the instant application, but rather teach the use of fungal (in particular *Aspegillus*) phytases.

In a non-limiting aspect, the present invention provides a method (and products therefrom) to produce composition comprised of the instant novel phytate-acting enzyme in combination with one or more additional enzymes that act on polysaccharides. Such polysaccharides can be selected from the group consisting of arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans, levan, fucoidan, carrageenan, galactocarolose, pectin, pectic acid, amylose, pullulan, glycogen, amylopectin, cellulose, carboxylmethylcellulose, hydroxypropylmethylcellulose, dextran, pustulan, chitin, agarose, keratan, chondroitin, dermatan, hyaluronic acid, alginic acid, and polysaccharides containing at least one aldose, ketose, acid or amine selected from the group consisting of erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine and neuraminic acid

In a particular aspect, the present invention provides a method (and products therefrom) to produce composition having a synergistic phytate hydrolyzing activity comprising one or more novel phytase molecules of the instant invention, a cellulase (including preferably but not exclusively a xylanase), optionally a protease, and optionally one or more additonal reagents. In preferred embodiments, such

combination treatments are serviceable in the treatment of foodstuffs, wood products, such as paper products, and as cleansing solutions and solids.

In one non-limiting exemplification, the instant phytase molecules are serviceable in combination with cellulosome components. It is known that cellulases of many cellulolytic bacteria are organized into discrete multienzyme complexes, called cellulosomes. The multiple subunits of cellulosomes are composed of numerous functional domains, which interact with each other and with the cellulosic substrate. One of these subunits comprises a distinctive new class of noncatalytic scaffolding polypeptide, which selectively integrates the various cellulase and xylanase subunits into the cohesive complex. Intelligent application of cellulosome hybrids and chimeric constructs of cellulosomal domains should enable better use of cellulosic biomass and may offer a wide range of novel applications in research, medicine and industry.

In another non-limiting exemplification, the instant phytase molecules are serviceable - either alone or in combination treatments - in areas of biopulping and biobleaching where a reduction in the use of environmentally harmful chemicals traditionally used in the pulp and paper industry is desired. Waste water treatment represents another vast application area where biological enzymes have been shown to be effective not only in colour removal but also in the bioconversion of potentially noxious substances into useful bioproducts.

In another non-limiting exemplification, the instant phytase molecules are serviceable for generating life forms that can provide at least one enzymatic activity - either alone or in combination treatments - in the treatment of digestive systems of organisms. Particularly relevant organisms to be treated include non-ruminant organisms. Specifically, it is appreciated that this approach may be performed alone or in combination with other biological molecules (for example, xylanases) to generate a recombinant host that expresses a plurality of biological molecules. It is also appreciated that the administration of the instant phytase molecules &/or recombinant hosts expressing the instant phytase molecules may be performed either alone or in

combination with other biological molecules, &/or life forms that can provide enzymatic activities in a digestive system - where said other enzymes and said life forms may be may recombinant or otherwise. For example, administration may be performed in combination with xylanolytic bacteria

For example, in addition to phytate, many organisms are also unable to adequately digest hemicelluloses. Hemicelluloses or xylans are major components (35%) of plant materials. For ruminant animals, about 50% of the dietary xylans are degraded, but only small amounts of xylans are degraded in the lower gut of nonruminant animals and humans. In the rumen, the major xylanolytic species are Butyrivibrio fibrisolvens and Bacteroides ruminicola. In the human colon, Bacteroides ovatus and Bacteroides fragilis subspecies "a" are major xylanolytic bacteria. Xylans are chemically complex, and their degradation requires multiple enzymes. Expression of these enzymes by gut bacteria varies greatly among species. Butyrivibrio fibrisolvens makes extracellular xylanases but Bacteroides species have cell-bound xylanase activity. Biochemical characterization of xylanolytic enzymes from gut bacteria has not been done completely. A xylosidase gene has been cloned from B. fibrosolvens 113. The data from DNA hybridizations using a xylanase gene cloned from B. fibrisolvens 49 indicate this gene may be present in other B. fibrisolvens strains. A cloned xylanase from Bact. ruminicola was transferred to and highly expressed in Bact. fragilis and Bact. uniformis. Arabinosidase and xylosidase genes from Bact. ovatus have been cloned and both activities appear to be catalyzed by a single, bifunctional, novel enzyme.

Accordingly, it is appreciated that the present phytase molecules are serviceable for 1) transferring into a suitable host (such as Bact. fragilis or Bact. uniformis); 2) achieving adequate expression in a resultant recombinant host; and 3) administering said recombinant host to organisms to improve the ability of the treated organisms to degrade phytate. Continued research in genetic and biochemical areas will provide knowledge and insights for manipulation of digestion at the gut level and improved understanding of colonic fiber digestion.

Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,624,678 (Bedford *et al.*), USPN 5,683,911 (Bodie *et al.*), USPN 5,720,971 (Beauchemin *et al.*), USPN 5,759,840 (Sung *et al.*), USPN 5,770,012 (Cooper), USPN 5,786,316 (Baeck *et al.*), USPN 5,817,500 (Hansen *et al.*), and journal articles (Jeffries, 1996; Prade, 1996; Bayer *et al.*, 1994; Duarte *et al.*, 1994; Hespell & Whitehead, 1990; Wong *et al.*, 1988), although these reference do not teach the inventive phytase molecules of the instant application, nor do they all teach the addition of phytase molecules in the production of foodstuffs, wood products, such as paper products, and as cleansing solutions and solids. In contrast, the instant invention teaches that phytase molecules – preferably the inventive phytase molecules of the instant application - may be added to the reagent(s) disclosed in order to obtain preparations having an additional phytase activity. Preferably, said reagent(s) the additional phytase molecules and will not inhibit each other, more preferably said reagent(s) the additional phytase molecules will have an overall additive effect, and more preferably still said reagent(s) the additional phytase molecules will have an overall synergistic effect.

In a non-limiting aspect, the present invention provides a method (and products therefrom) for enhancement of phytate phosphorus utilization and treatment and prevention of tibial dyschondroplasia in animals, particularly poultry, by administering to animals a feed composition containing a hydroxylated vitamin D_3 derivative. The vitamin D_3 derivative is preferably administered to animals in feed containing reduced levels of calcium and phosphorus for enhancement of phytate phosphorus utilization. Accordingly, the vitamin D_3 derivative is preferably administered in combination with novel phytase molecules of the instant invention for further enhancement of phytate phosphorus utilization. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,516,525 (Edwards *et al.*) and USPN 5,366,736 (Edwards *et al.*), USPN

5,316,770 (Edwards *et al.*) although these reference do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method (and products therefrom) to obtain foodstuff that 1) comprises phytin that is easily absorbed and utilized in a form of inositol in a body of an organism; 2) that is capable of reducing phosphorus in excrementary matter; and 3) that is accordingly useful for improving environmental pollution. Said foodstuff is comprised of an admixture of a phytin-containing grain, a lactic acid-producing microorganism, and a novel phytase molecule of the instant invention. In a preferred embodiment, said foodstuff is produced by compounding a phytin- containing grain (preferably, *e.g.* rice bran) with an effective microbial group having an acidophilic property, producing lactic acid, without producing butyric acid, free from pathogenicity, and a phytase. Examples of an effective microbial group include *e.g. Streptomyces sp.* (ATCC 3004) belonging to the group of actinomyces and *Lactobacillus sp.* (IFO 3070) belonging to the group of lactobacilli. Further, a preferable amount of addition of an effective microbial group is 0.2 wt. % in terms of bacterial body weight based on a grain material. Furthermore, the amount of the addition of the phytase is preferably 1-2 wt. % based on the phytin in the grain material. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 08205785 (Akahori *et al.*), although references in the publicly available literature do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method for improving the solubility of vegetable proteins. More specifically, the invention relates to methods for the solubilization of proteins in vegetable protein sources, which methods comprise treating the vegetable protein source with an efficient amount of one or more phytase enzymes – including phytase molecules of the instant invention - and treating the vegetable protein source with an efficient amount of one or more proteolytic enzymes. In another aspect, the invention provides animal feed additives

comprising a phytase and one or more proteolytic enzymes. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes EP 0756457 (WO 9528850 A1) (Nielsen and Knap), although references in the publicly available literature do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method of producing a plant protein preparation comprising dispersing vegetable protein source materials in water at a pH in the range of 2 to 6 and admixing phytase molecules of the instant invention therein. The acidic extract containing soluble protein is separated and dried to yield a solid protein of desirable character. One or more proteases can also be used to improve the characteristics of the protein. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 3,966,971 (Morehouse *et al.*), although references in the publicly available literature do not teach the inventive molecules of the instant application.

In a non-limiting aspect, the present invention provides a method (and products thereof) to activate inert phosphorus in soil and/or compost, to improve the utilization rate of a nitrogen compound, and to suppress propagation of pathogenic molds by adding three reagents, phytase, saponin and chitosan, to the compost. In a non-limiting embodiment the method can comprise treating the compost by 1) adding phytase-containing microorganisms in media – preferably recombinant hosts that overexpress the novel phytase molecules of the instant invention - *e.g.* at 100 ml media/100 kg wet compost; 2) alternatively also adding a phytase-containing plant source - such as wheat bran - *e.g.* at 0.2 to 1 kg/100 kg wet compost; 3) adding a saponin-containing source - such as peat, mugworts and yucca plants – *e.g.* at 0.5 to 3.0g/kg ; 4) adding chitosan-containing materials – such as pulverized shells of shrimps, crabs, etc. – *e.g.* at 100 to 300g/kg wet compost. In another non-limiting embodiment, recombinant sources the three reagents, phytase, saponin, and chitosan,

are used. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 07277865 (Toya Taisuke), although references in the publicly available literature do not teach the inventive molecules of the instant application.

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type have at least 10, preferably at least 15, and even more preferably at least 30 bases and may contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns.

The present invention provides methods for identifying nucleic acid molecules that encode members of the phytase polypeptide family in addition to SEQ ID NO:1. In these methods, a sample, *e.g.*, a nucleic acid library, such as a cDNA library, that contains a nucleic acid encoding a phytase polypeptide is screened with a phytase-specific probe, *e.g.*, a phytase-specific nucleic acid probe. Phytase-specific nucleic acid probes are nucleic acid molecules (*e.g.*, molecules containing DNA or RNA nucleotides, or combinations or modifications thereof) that specifically hybridize to nucleic acids encoding phytase polypeptides, or to complementary sequences thereof. The term "phytase-specific probe," in the context of this method of invention, refers to probes that bind to nucleic acids encoding phytase polypeptides, or to complementary sequences thereof, to a detectably greater extent than to nucleic acids encoding other enzymes, or to complementary sequences thereof.

The invention facilitates production of phytase-specific nucleic acid probes. Methods for obtaining such probes can be designed based on the amino acid sequences shown in Figure 1. The probes, which can contain at least 12, *e.g.*, at least 15, 25, 35, 50, 100, or 150 nucleotides, can be produced using any of several standard

methods (see, *e.g.*, Ausubel *et al.*, *supra*). For example, preferably, the probes are generated using PCR amplification methods. In these methods, primers are designed that correspond to phytase-conserved sequences (see Figure 1), which can include phytase-specific amino acids, and the resulting PCR product is used as a probe to screen a nucleic acid library, such as a cDNA library.

This invention can be used to isolate nucleic acid sequences substantially similar to the isolated nucleic acid molecule encoding an phytase enzyme disclosed in Figure 1 (SEQ ID NO:1). Isolated nucleic acid sequences are substantially similar if: (i) they are capable of hybridizing under stringent conditions, hereinafter described, to SEQ ID NO:1; or (ii) they encode a phytase polypeptide as set forth in SEQ ID NO:2 due to the degeneracy of the genetic code (*e.g.*,degenerate to SEQ ID NO:1).

Degenerate DNA sequences encode the amino acid sequence of SEQ ID NO:2, but have variations in the nucleotide coding sequences. As used herein, "substantially similar" refers to the sequences having similar identity to the sequences of the instant invention. The nucleotide sequences that are substantially similar can be identified by hybridization or by sequence comparison. Enzyme sequences that are substantially similar can be identified by one or more of the following: proteolytic digestion, gel electrophoresis and/or microsequencing.

One means for isolating a nucleic acid molecule encoding a phytase enzyme is to probe a genomic gene library with a natural or artificially designed probe using art recognized procedures (see, *e.g.*, Ausubel *et al.*, *supra*). It is appreciated to one skilled in the art that SEQ ID NO:1, or fragments thereof (comprising at least 15 contiguous nucleotides), is a particularly useful probe. Other particular useful probes for this purpose are hybridizable fragments to the sequences of SEQ ID NO:1 (*i.e.*, comprising at least 15 contiguous nucleotides).

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of

catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of DNA from other animal sources or to screen such sources for related sequences.

With respect to nucleic acid sequences which hybridize to specific nucleic acid sequences disclosed herein, hybridization may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions. As an example of oligonucleotide hybridization, a polymer membrane containing immobilized denatured nucleic acid is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH_2PO_4, pH 7.0, 5.0 mM Na_2EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2 X 10^7 cpm (specific activity 4-9 X 10^8 cpm/ug) of ^{32}P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na_2EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at Tm-10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

The nucleic acid molecules of the invention can be used as templates in standard methods for production of phytase gene products (*e.g.*, phytase RNAs and phytase polypeptides). In addition, the nucleic acid molecules that encode phytase polypeptides (and fragments thereof) and related nucleic acids - such as (1) nucleic acids containing sequences that are complementary to, or that hybridize to, nucleic acids encoding phytase polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides); and (2) nucleic acids containing sequences that hybridize to sequences that are complementary to nucleic acids encoding phytase polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides) - can be used in methods focused on their hybridization properties. For example, as is described in further detail herein, such nucleic acid molecules can be used in the following methods: PCR methods for synthesizing phytase nucleic acids,

methods for detecting the presence of a phytase nucleic acid in a sample, screening methods for identifying nucleic acids encoding new phytase family members. Hybridization-based uses include Southern-type, Northern-type, RNA protection, and any hybridization procedure were a nucleic acid is used as a hybridization partner.

Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention. Accordingly, fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Size separation of the cleaved fragments is generally performed using 8 percent polyacrylamide gel as described in the literature (*e.g.* by Goeddel *et al.*, 1980).

This invention provides enzymes, as well as fragments, other derivatives, and analogs thereof, and the corresponding nucleotides for use in directed evolution. The discovery and use of a plurality of templates as disclosed herein may significantly increase the potential yield of directed evolution in comparison to the directed evolution of a single template protein. Hence, the need for discovery is based on the premise that nature provides a wealth of potentially unattainable or unpredictable features in distinct but related members of molecular groupings, and that the exploitation of these features may greatly facilitate directed evolution. Thus, in one aspect, related but distinct molecules may serve as unique starting templates for the directed evolution of a desired characteristic. In another aspect, they may serve as repositories of structure-function information including, but not limited to, a variety of consensus motifs. Both utilities help to obviate the logistically impractical task of at-once exploring an overly wide range of mutational permutations on any given molecule. For example, the full range of mutational permutations on a 100 amino acid protein includes over 10^{130} possibilities (assuming there are 20 amino acid possibilities at each position), a number too large for practical consideration.

Accordingly, particularly because of logistical and technical constraints, it is a desirable approach - in performing "directed evolution" – to discover and to make use

of a plurality of related starting templates that have pre-evolved differences. These templates can then be subjected to a variety of mutagenic manipulations including, by way of non-limiting exemplification, DNA mutagenesis and combinatorial enzyme development, an approach that is further elaborated in co-pending USPN 5,830,696 (Short *et al.*).

The enzyme activities of the novel molecules generated can then be screened by a variety of methods including, by way of non-limiting exemplification: a) molecular biopanning; b) recombinant clone screening; and c) extract screening.

This invention provides enzymes, as well as fragments, other derivatives, and analogs thereof, and cells expressing them that can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the enzymes corresponding to a sequence of the present invention can be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985, pp. 77-96).

Techniques described for the production of single chain antibodies (USPN 4,946,778 Ladner *et al.*) can be adapted to produce single chain antibodies to immunogenic enzyme products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic enzyme products of this invention.

Antibodies generated against the enzyme of the present invention may be used in screening for similar enzymes from other organisms and samples. Such screening techniques are known in the art. Antibodies may also be employed as a probe to screen gene libraries generated from this or other organisms to identify this or cross reactive activities.

Isolation and purification of polypeptides produced in the systems described above can be carried out using conventional methods, appropriate for the particular system. For example, preparative chromatography and immunological separations employing antibodies, such as monoclonal or polyclonal antibodies, can be used.

As is mentioned above, antigens that can be used in producing phytase-specific antibodies include phytase polypeptides, *e.g.*, any of the phytase shown in Figures 1 polypeptide fragments. The polypeptide or peptide used to immunize an animal can be obtained by standard recombinant, chemical synthetic, or purification methods. As is well known in the art, in order to increase immunogenicity, an antigen can be conjugated to a carrier protein. Commonly used carriers include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit). In addition to such carriers, well known adjuvants can be administered with the antigen to facilitate induction of a strong immune response.

Phytase-specific polyclonal and monoclonal antibodies can be purified, for example, by binding to, and elution from, a matrix containing a phytase polypeptide, *e.g.*, the phytase polypeptide (or fragment thereof) to which the antibodies were raised. Additional methods for antibody purification and concentration are well

known in the art and can be practiced with the phytase-specific antibodies of the invention (see, *e.g.*, Coligan *et al.*, 1996).

Anti-idiotype antibodies corresponding to phytase-specific antigens are also included in the invention, and can be produced using standard methods. These antibodies are raised to phytase-specific antibodies, and thus mimic phytase-specific epitopes.

This invention also includes additonal uses of fragments of the phytase polypeptides that retain at least one phytase-specific activity or epitope. Phytase activity can be assayed by examining the catalysis of phytate to inositol and free phosphate. Such fragments can easily be identified by comparing the sequences of phytases found in Figure 1.

In a non-limiting exemplification, a phytase polypeptide fragment containing, *e.g.*, at least 8-10 amino acids can be used as an immunogen in the production of phytase-specific antibodies. The fragment can contain, for example, an amino acid sequence that is conserved in phytases, and this amino acid sequence can contain amino acids that are conserved in phytases. In another non-limiting exemplification,, the above-described phytase fragments can be used in immunoassays, such as ELISAs, to detect the presence of phytase-specific antibodies in samples.

Various methods to make the transgenic animals of the subject invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in U.S. Pat. No. 4,873,191. In yet another such

method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Pat No. 5,162,215. If micro-injection is to be used with avian species, however, a published procedure by Love *et al.*, (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half hours after the laying of the previous laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

The "non-human animals" of the invention bovine, porcine, ovine and avian animals (*e.g.*, cow, pig, sheep, chicken). The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for micro-injection. The use of zygotes as is target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

The term "transgenic" is used to describe an animal which includes exogenous genetic material within all of its cells. A "transgenic" animal can be produced by cross-breeding two chimeric animals which include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will be transgenic *i.e.*, animals which include the exogenous genetic material within all of their cells in both alleles, 50% of the resulting animals will include the exogenous genetic material within one allele and 25% will include no exogenous genetic material.

In the microinjection method useful in the practice of the subject invention, the transgene is digested and purified free from any vector DNA, *e.g.*, by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV) , Moloney leukemia virus (MLV), and herpes virus, as well as those from the genes encoding metallothionin, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase. Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can also be employed. When the animals to be made transgenic are avian, preferred promoters include those for the chicken b-globin gene, chicken lysozyme gene, and avian leukosis virus. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R., Proc. Natl. Acad. Sci USA 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, *et al.* (1986) in Manipulating the Mouse Embryo, Cold

Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner, *et al.*, Proc. Natl. Acad. Sci. USA 82: 6927-6931, 1985; Van der Putten, *et al.*, Proc. Natl. Acad. Sci USA 82: 6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, EMBO J. 6: 383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (D. Jahner *et al.*, Nature 298: 623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder may contain various retro viral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (D. Jahner *et al.*, *supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (M. J. Evans *et al.*, Nature 292:154-156, 1981; M. O. Bradley *et al.*, Nature 309:255-258, 1984; Gossler, *et al.*, Proc. Natl. Acad. Sci USA 83:9065-9069, 1986; and Robertson *et al.*, Nature 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. (For review see Jaenisch, R., Science 240:1468-1474, 1988).

"Transformed" means a cell into which (or into an ancestor of which) has been introduced, by means of recombinant nucleic acid techniques, a heterologous nucleic acid molecule. "Heterologous" refers to a nucleic acid sequence that either originates

from another species or is modified from either its original form or the form primarily expressed in the cell.

"Transgene" means any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism (*i.e.*, either stably integrated or as a stable extrachromosomal element) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence which is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode phytases or polypeptides having phytase activity, and include polynucleotides, which may be expressed in a transgenic non-human animal. The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" as used herein, refers to the targeted disruption of a gene *in vivo* with complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out."

The transgene to be used in the practice of the subject invention is a DNA sequence comprising a sequence coding for a phytase or a polypeptide having phytase activity. In a one embodiment, a polynucleotide having a sequence as set forth in SEQ ID NO:1 or a sequence encoding a polypeptide having a sequence as set forth in SEQ ID NO:2 is the transgene as the term is defined herein. Where appropriate, DNA sequences that encode proteins having phytase activity but differ in nucleic acid

sequence due to the degeneracy of the genetic code may also be used herein, as may truncated forms, allelic variants and interspecies homologues.

After an embryo has been microinjected, colonized with transfected embryonic stem cells or infected with a retrovirus containing the transgene (except for practice of the subject invention in avian species which is addressed elsewhere herein) the embryo is implanted into the oviduct of a pseudopregnant female. The consequent progeny are tested for incorporation of the transgene by Southern blot analysis of blood or tissue samples using transgene specific probes. PCR is particularly useful in this regard. Positive progeny (G0) are crossbred to produce offspring (G1) which are analyzed for transgene expression by Northern blot analysis of tissue samples.

Thus, the present invention includes methods for increasing the phosphorous uptake in the transgenic animal and/or decreasing the amount of polltant in the manure of the transgenic organism by about 15%, typically about 20%, and more typically about 20% to about 50%.

The animals contemplated for use in the practice of the subject invention are those animals generally regarded as domesticated animals including pets (*e.g.*, canines, felines, avian species *etc.*) and those useful for the processing of food stuffs, *i.e.*, avian such as meat bred and egg laying chicken and turkey, ovine such as lamb, bovine such as beef cattle and milk cows, piscine and porcine. For purposes of the subject invention, these animals are referred to as "transgenic" when such animal has had a heterologous DNA sequence, or one or more additional DNA sequences normally endogenous to the animal (collectively referred to herein as "transgenes") chromosomally integrated into the germ cells of the animal. The transgenic animal (including its progeny) will also have the transgene fortuitously integrated into the chromosomes of somatic cells.

In some instances it may be advantageous to deliver and express a phytase sequence of the invention locally (*e.g.*, within a particular tissue or cell type). For example, local expression of a phytase or digestive enzyme in the gut of an animal

will assist in the digestion and uptake of, for example, phytate and phosporous, respectively. The nucleic sequence may be directly delivered to the salivary glands, tissue and cells and/or to the epithelial cells lining the gut, for example. Such delivery methods are known in the art and include electroporation, viral vectors and direct DNA uptake. Any polypeptide having phytase activity can be utilized in the methods of the invention (*e.g.*, those specficially described herein, as well as those described in other sections of the invention).

For example, a nucleic acid constructs of the present invention will comprise nucleic acid molecules in a form suitable for uptake into target cells within a host tissue. The nucleic acids may be in the form of bare DNA or RNA molecules, where the molecules may comprise one or more structural genes, one or more regulatory genes, antisense strands, strands capable of triplex formation, or the like. Commonly, the nucleic acid construct will include at least one structural gene under the transcriptional and translational control of a suitable regulatory region. More usually, nucleic acid constructs of the present invention will comprise nucleic acids incorporated in a delivery vehicle to improve transfection efficiency, wherein the delivery vehicle will be dispersed within larger particles comprising a dried hydrophilic excipient material.

One such delivery vehicles comprises viral vectors, such as retroviruses, adenoviruses, and adeno-associated viruses, which have been inactivated to prevent self-replication but which maintain the native viral ability to bind a target host cell, deliver genetic material into the cytoplasm of the target host cell, and promote expression of structural or other genes which have been incorporated in the particle. Suitable retrovirus vectors for mediated gene transfer are described in Kahn *et al.* (1992) CIRC. RES. 71:1508-1517, the disclosure of which is incorporated herein by reference. A suitable adenovirus gene delivery is described in Rosenfeld *et al.* (1991) SCIENCE 252:431-434, the disclosure of which is incorporated herein by reference. Both retroviral and adenovirus delivery systems are described in Friedman (1989)

SCIENCE 244:1275-1281, the disclosure of which is also incorporated herein by reference.

A second type of nucleic acid delivery vehicle comprises liposomal transfection vesicles, including both anionic and cationic liposomal constructs. The use of anionic liposomes requires that the nucleic acids be entrapped within the liposome. Cationic liposomes do not require nucleic acid entrapment and instead may be formed by simple mixing of the nucleic acids and liposomes. The cationic liposomes avidly bind to the negatively charged nucleic acid molecules, including both DNA and RNA, to yield complexes which give reasonable transfection efficiency in many cell types. See, Farhood *et al.* (1992) BIOCHEM. BIOPHYS. ACTA. 1111:239-246, the disclosure of which is incorporated herein by reference. A particularly preferred material for forming liposomal vesicles is lipofectin which is composed of an equimolar mixture of dioleylphosphatidyl ethanolamine (DOPE) and dioleyloxypropyl-triethylammonium (DOTMA), as described in Felgner and Ringold (1989) NATURE 337:387-388, the disclosure of which is incorporated herein by reference.

It is also possible to combine these two types of delivery systems. For example, Kahn *et al.* (1992), *supra.*, teaches that a retrovirus vector may be combined in a cationic DEAE-dextran vesicle to further enhance transformation efficiency. It is also possible to incorporate nuclear proteins into viral and/or liposomal delivery vesicles to even further improve transfection efficiencies. See, Kaneda *et al.* (1989) SCIENCE 243:375-378, the disclosure of which is incorporated herein by reference.

In another embodiment, a digestive aid containing an enzyme either as the sole active ingredient or in combination with one or more other agents and/or enzymes is provided (as described in co-pending application U.S. Serial No. 09/580,937, entitled "Dietary Aids and Methods of Use Thereof," filed May 25, 2000, the disclosure of which is incorporated herein by reference in its entirety). The use of enzymes and other agents in digestive aids of livestock or domesticated animals not only improves

the animal's health and life expectancy but also assists in increasing the health of livestock and in the production of foodstuffs from livestock.

Currently, some types of feed for livestock (*e.g.*, certain poultry feed) are highly supplemented with numerous minerals (*e.g.*, inorganic phosphorous), enzymes, growth factors, drugs, and other agents for delivery to the livestock. These supplements replace many of the calories and natural nutrients present in grain, for example.

By reducing or eliminating the inorganic phosphorous supplement and other supplements (*e.g.*, trace mineral salts, growth factors, enzymes, antibiotics) from the feed itself, the feed would be able to carry more nutrient and energy. Accordingly, the remaining diet would contain more usable energy. For example, grain-oilseed meal diets generally contain about 3,200 kcal metabolizable energy per kilogram of diet, and mineral salts supply no metabolizable energy. Removal of the unneeded minerals and substitution with grain would therefore increase the usable energy in the diet. Thus, the invention can be differentiated over commonly used phytase containing feed. For example, in one embodiment, a biocompatible material is used that is resistant to digestion by the gastrointestinal tract of an organism.

In many organisms, including, for example, poultry or birds such as, for example, chickens, turkeys, geese, ducks, parrots, peacocks, ostriches, pheasants, quail, pigeons, emu, kiwi, loons, cockatiel, cockatoo, canaries, penguins, flamingoes, and dove, the digestive tract includes à gizzard which stores and uses hard biocompatible objects (*e.g.*, rocks and shells from shell fish) to help in the digestion of seeds or other feed consumed by a bird. A typical digestive tract of this general family of organisms, includes the esophagus which contains a pouch, called a crop, where food is stored for a brief period of time. From the crop, food moves down into the true stomach, or *proventriculus*, where hydrochloric acid and pepsin starts the process of digestion. Next, food moves into the gizzard, which is oval shaped and thick walled with powerful muscles. The chief function of the gizzard is to grind or crush food particles - a process which is aided by the bird swallowing small amounts

of fine gravel or grit. From the gizzard, food moves into the duodenum. The small intestine of birds is similar to mammals. There are two blind pouches or *ceca*, about 4-6 inches in length at the junction of the small and large intestine. The large intestine is short, consisting mostly of the rectum about 3-4 inches in length. The rectum empties into the *cloaca* and feces are excreted through the vent.

Hard, biocompatible objects consumed (or otherwise introduced) and presented in the gizzard provide a useful vector for delivery of various enzymatic, chemical, therapeutic and antibiotic agents. These hard substances have a life span of a few hours to a few days and are passed after a period of time. Accordingly, the invention provides coated, impregnated (*e.g.*, impregnated matrix and membranes) modified dietary aids for delivery of useful digestive or therapeutic agents to an organism. Such dietary aids include objects which are typically ingested by an organism to assist in digestion within the gizzard (*e.g.*, rocks or grit). The invention provides biocompatible objects that have coated thereon or impregnated therein agents useful as a digestive aid for an organism or for the delivery of a therapeutic or medicinal agent or chemical.

In a first embodiment, the invention provides a dietary aid, having a biocompatible composition designed for release of an agent that assists in digestion, wherein the biocompatible composition is designed for oral consumption and release in the digestive tract (*e.g.*, the gizzard) of an organism. "Biocompatible" means that the substance, upon contact with a host organism (*e.g.*, a bird), does not elicit a detrimental response sufficient to result in the rejection of the substance or to render the substance inoperable. Such inoperability may occur, for example, by formation of a fibrotic structure around the substance limiting diffusion of impregnated agents to the host organism therein or a substance which results in an increase in mortality or morbidity in the organism due to toxicity or infection. A biocompatible substance may be non-biodegradable or biodegradable. In one embodiment, the biocompatible composition is resistant to degradation or digestion by the gastrointestinal tract. In

another embodiment, the biocompatible composition has the consistency of a rock or stone.

A non-biodegradable material useful in the invention is one that allows attachment or impregnation of a dietary agent. Such non-biodegradable materials include, for example, thermoplastics, such as acrylic, modacrylic, polyamide, polycarbonate, polyester, polyethylene, polypropylene, polystyrene, polysulfone, polyethersulfone, and polyvinylidene fluoride. Elastomers are also useful materials and include, for example, polyamide, polyester, polyethylene, polypropylene, polystyrene, polyurethane, polyvinyl alcohol and silicone (*e.g.*, silicone based or containing silica). The invention provides that the biocompatible composition can contain a plurality of such materials, which can be, *e.g.*, admixed or layered to form blends, copolymers or combinations thereof.

As used herein, a "biodegradable" material means that the composition will erode or degrade *in vivo* to form smaller chemical species. Degradation may occur, for example, by enzymatic, chemical or physical processes. Suitable biodegradable materials contemplated for use in the invention include poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polycarbonate, polycyanoacrylate, polyurethanes, polyacrylate. Such materials can be admixed or layered to form blends, copolymers or combinations thereof.

It is contemplated that a number different biocompatible substances may be ingested or otherwise provided to the same organism simultaneously, or in various combinations (*e.g.*, one material before the other). In addition, the biocompatible substance may be designed for slow passage through the digestive tract. For example, large or fatty substances tend to move more slowly through the digestive tract, accordingly, a biocompatible material having a large size to prevent rapid passing in the digestive tract can be used. Such large substances can be a combination of non-biodegradable and biodegradable substances. For example, a small non-biodegradable substance can be encompassed by a biodegradable substance such that over a period of

time the biodegradable portion will be degraded allowing the non-biodegradable portion to pass through the digestive trace. In addition, it is recognized that any number of flavorings can be provided to the biocompatible substance to assist in consumption.

Any number of agents alone or in combination with other agents can be coated on the biocompatible substance including polypeptides (*e.g.*, enzymes, antibodies, cytokines or therapeutic small molecules), and antibiotics, for example. Examples of particular useful agents are listed in Table 1 and 2, below. It is also contemplated that cells can be encapsulated into the biocompatible material of the invention and used to deliver the enzymes or therapeutics. For example, porous substances can be designed that have pores large enough for cells to grow in and through and that these porous materials can then be taken into the digestive tract. For example, the biocompatible substance can be comprised of a plurality of microfloral environments (*e.g.*, different porosity, pH etc.) that provide support for a plurality of cell types. The cells can be genetically engineered to deliver a particular drug, enzyme or chemical to the organism. The cells can be eukaryotic or prokaryotic.

Table 1

Treatment Class	Chemical	Description
Antibiotics	Amoxycillin and Its Combination Mastox Injection (Amoxycillin and Cloxacillin)	Treatment Against Bacterial Diseases Caused By Gram + and Gram – Bacteria
	Ampicillin and Its Combination Biolox Injection (Ampicillin and Cloxacillin)	Treatment Against Bacterial Diseases Caused By Gram + And Gram – Bacteria.
	Nitrofurazone + Urea Nefrea Bolus	Treatment Of Genital Infections
	Trimethoprim + Sulphamethoxazole Trizol Bolus	Treatment Of Respiratory Tract Infections, Gastro Intestinal Tract Infections, Urino- Genital Infections.
	Metronidazole and Furazolidone Metofur Bolus	Treatment Of Bacterial And Protozoal Diseases.

	Phthalylsulphathiazole, Pectin and Kaolin Pectolin Bolus Suspension	Treatment Of Bacterial And Non-Specific Diarrhoea, Bacillary Dysentry And Calf Scours.
Antihelmintics	Ectoparasiticide Germex Ointment (Gamma Benzene Hexachloride, Proflavin Hemisulphate and Cetrimide)	Ectoparasiticide and Antiseptic
	Endoparasiticides > Albendazole and Its Combination Alben (Albendazole) Suspension (Albendazole 2.5%) Plus Suspension (Albendazole 5%) Forte Bolus (Albendazole 1.5 Gm.) Tablet (Albendazole 600 Mg.) Powder(Albendazole 5%, 15%)	Prevention And Treatment Of Roundworm, Tapeworm and Fluke Infestations
	Alpraz (Albendazole and Praziquantel)Tablet	Prevention And Treatment Of Roundworm and Tapeworm Infestation In Canines and Felines.
	Oxyclozanide and Its Combination Clozan (Oxyclozanide) Bolus, Suspension	Prevention and Treatment Of Fluke Infestations
	Tetzan (Oxyclozanide and Tetramisole Hcl) Bolus, Suspension	Prevention and Treatment Of Roundworm and Fluke Infestations
	Fluzan (Oxyclozanide and Levamisole Hcl) Bolus, Suspension	Prevention and Treatment Of Roundworm Infestations and Increasing Immunity
	Levamisole Nemasol Injection Wormnil Powder	Prevention and Treatment Of Roundworm Infestations and Increasing Immunity.
	Fenbendazole Fenzole Tablet (Fenbendazole150 Mg.) Bolus (Fenbendazole 1.5 Gm.) Powder (Fenbendazole 2.5% W/W)	Prevention And Treatment of Roundworm and Tapeworm Infestations

Tonics	Vitamin B Complex, Amino Acids and Liver Extract Heptogen Injection	Treatment Of Anorexia, Hepatitis, Debility, Neuralgic Convulsions Emaciation and Stunted Growth.
	Calcium Levulinate With Vit.B_{12} and Vit D_3 Hylactin Injection	Prevention and treatment of hypocalcaemia, supportive therapy in sick conditions (especially hypothermia) and treatment of early stages of rickets.
Animal Feed Supplements	Essential Minerals, Selenium and Vitamin E Gynolactin Bolus	Treatment Of Anoestrus Causing Infertility and Repeat Breeding In Dairy Animals and Horses.
	Essential Minerals, Vitamin E, and Iodine Hylactin Powder	Infertility, Improper Lactation, Decreased Immunity, Stunted Growth and Debility.
	Essential Electrolytes With Vitamin C Electra – C Powder	Diarrhoea, Dehydration, Prior to and after Transportation, In Extreme temperatures (High Or Low) and other Conditions of stress.
	Pyrenox Plus (Diclofenac Sodium + Paracetamol) Bolus, Injection.	Treatment Of Mastitis, Pyrexia Post Surgical Pain and Inflammation, Prolapse Of Uterus, Lameness and Arthritis.

Table 2.

Therapeutic Formulations

Product	Description
Acutrim® (phenylpropanolamine)	Once-daily appetite suppressant tablets.
The Baxter® Infusor	For controlled intravenous delivery of anticoagulants, antibiotics, chemotherapeutic agents, and other widely used drugs.
Catapres-TTS® (clonidine transdermal therapeutic system)	Once-weekly transdermal system for the treatment of hypertension.
Covera HS™ (verapamil hydrochloride)	Once-daily Controlled-Onset Extended-Release (COER-24) tablets for the treatment of hypertension and angina pectoris.
DynaCirc CR® (isradipine)	Once-daily extended release tablets for the treatment of hypertension.

Efidac 24® (chlorpheniramine maleate)	Once-daily extended release tablets for the relief of allergy symptoms.
Estraderm® (estradiol transdermal system)	Twice-weekly transdermal system for treating certain postmenopausal symptoms and preventing osteoporosis
Glucotrol XL® (glipizide)	Once-daily extended release tablets used as an adjunct to diet for the control of hyperglycemia in patients with non-insulin-dependent diabetes mellitus.
IVOMEC SR® Bolus (ivermectin)	Ruminal delivery system for season-long control of major internal and external parasites in cattle.
Minipress XL® (prazosin)	Once-daily extended release tablets for the treatment of hypertension.
NicoDerm® CQ™ (nicotine transdermal system)	Transdermal system used as a once-daily aid to smoking cessation for relief of nicotine withdrawal symptoms.
Procardia XL® (nifedipine)	Once-daily extended release tablets for the treatment of angina and hypertension.
Sudafed® 24 Hour (pseudoephedrine)	Once-daily nasal decongestant for relief of colds, sinusitis, hay fever and other respiratory allergies.
Transderm-Nitro® (nitroglycerin transdermal system)	Once-daily transdermal system for the prevention of angina pectoris due to coronary artery disease.
Transderm Scop® (scopolamin transdermal system)	Transdermal system for the prevention of nausea and vomiting associated with motion sickness.
Volmax (albuterol)	Extended release tablets for relief of bronchospasm in patients with reversible obstructive airway disease.
Actisite®	(tetracycline hydrochloride) Periodontal fiber used as an adjunct to scaling and root planing for reduction of pocket depth and bleeding on probing in patients with adult periodontitis.
ALZET®	Osmotic pumps for laboratory research.
Amphotec® (amphotericin B cholesteryl sulfate complex for injection)	AMPHOTEC® is a fungicidal treatment for invasive aspergillosis in patients where renal impairment or unacceptable toxicity precludes use of amphotericin B in effective doses and in patients with invasive aspergillosis where prior amphotericin B therapy has failed.
BiCitra® (sodium citrate and citric acid)	Alkalinizing agent used in those conditions where long-term maintenance of alkaline urine is desirable.

Ditropan® (oxybutynin chloride)	For the relief of symptoms of bladder instability associated with uninhibited neurogenic or reflex neurogenic bladder (*i.e.*, urgency, frequency, urinary leakage, urge incontinence, dysuria).
Ditropan® XL (oxybutynin chloride)	is a once-daily controlled-release tablet indicated for the treatment of overactive bladder with symptoms of urge urinary incontinence, urgency and frequency.
DOXIL® (doxorubicin HCl liposome injection)	
Duragesic® (fentanyl transdermal system) CII	72-hour transdermal system for management of chronic pain in patients who require continuous opioid analgesia for pain that cannot be managed by lesser means such as acetaminophen-opioid combinations, non-steroidal analgesics, or PRN dosing with short-acting opioids.
Elmiron® (pentosan polysulfate sodium)	Indicated for the relief of bladder pain or discomfort associated with interstitial cystitis.
ENACT AirWatch™	An asthma monitoring and management system.
Ethyol® (amifostine)	Indicated to reduce the cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian cancer or non-small cell lung cancer. Indicated to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer, where the radiation port includes a substantial portion of the parotid glands.
Mycelex® Troche (clotrimazole)	For the local treatment of oropharyngeal candidiasis. Also indicated prophylactically to reduce the incidence of oropharyngeal candidiasis in patients immunocompromised by conditions that include chemotherapy, radiotherapy, or steroid therapy utilized in the treatment of leukemia, solid tumors, or renal transplantation.
Neutra-Phos® (potassium and sodium phosphate)	a dietary/nutritional supplement
PolyCitra® -K Oral Solution and PolyCitra® -K Crystals (potassium citrate and citric acid)	Alkalinizing agent useful in those conditions where long-term maintenance of an alkaline urine is desirable, such as in patents with uric acid and cystine calculi of the urinary tract, especially when the administration of sodium salts is undesirable or contraindicated
PolyCitra® -K Syrup and LC (tricitrates)	Alkalinizing agent useful in those conditions where long-term maintenance of an alkaline urine is desirable, such as in patients with uric acid and cystine calculi of the urinary tract.
Progestasert® (progesterone)	Intrauterine Progesterone Contraceptive System

Testoderm® Testoderm® with Adhesive and Testoderm® TTS CIII	Testosterone Transdermal System The Testoderm® products are indicated for replacement therapy in males for conditions associated with a deficiency or absence of endogenous testosterone: (1) Primary hypogonadism (congenital or acquired) or (2) Hypogonadotropic hypogonadism (congenital or acquired).
Viadur™ (leuprolide acetate implant)	Once-yearly implant for the palliative treatment of prostate cancer

Certain agents can be designed to become active or in activated under certain conditions (*e.g.*, at certain pH's, in the presence of an activating agent etc.). In addition, it may be advantageous to use pro-enzymes in the compositions of the invention. For example, a pro-enzymes can be activated by a protease (*e.g.*, a salivary protease that is present in the digestive tract or is artificially introduced into the digestive tract of an organism). It is contemplated that the agents delivered by the biocompatible compositions of the invention can be activated or inactivated by the addition of an activating agent which may be ingested by, or otherwise delivered to, the organism. Another mechanism for control of the agent in the digestive tract is an environment sensitive agent that is activated in the proper digestive compartment. For example, an agent may be inactive at low pH but active at neutral pH. Accordingly, the agent would be inactive in the gut but active in the intestinal tract. Alternatively, the agent can become active in response to the presence of a microorganism specific factor (*e.g.*, microorganisms present in the intestine).

In summary, the potential benefits of the present invention include, for example, (1) reduction in or possible elimination of the need for mineral supplements (*e.g.*, inorganic phosphorous supplements), enzymes, or therapeutic drugs for animal (including fish) from the daily feed or grain thereby increasing the amount of calories and nutrients present in the feed, and (2) increased health and growth of domestic and non-domestic animals including, for example, poultry, porcine, bovine, equine, canine, and feline animals.

A large number of enzymes can be used in the methods and compositions of the present invention. These enzymes include enzymes necessary for proper digestion of consumed foods, or for proper metabolism, activation or derivation of chemicals, prodrugs or other agents or compounds delivered to the animal via the digestive tract. Examples of enzymes that can be delivered or incorporated into the compositions of the invention, include, for example, feed enhancing enzymes selected from the group consisting of α-galactosidases, β-galactosidases, in particular lactases, phytases, β-glucanases, in particular endo-β-1,4-glucanases and endo-β-1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4-β-galactosidases and arabinogalactan endo-1,3-β-galactosidases, endoglucanases, in particular endo-1,2-β-glucanase, endo-1,3-α-glucanase, and endo-1,3-β-glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan-α-rhamnosidase, pectate lyases, and α-galacturonisidases, mannanases, β-mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases. Phytases as set forth in SEQ ID NO:1 and 2 and in Table 3 below are preferred. The sequences described in Table 3 are SEQ ID NO:1 and 2 having the amino acid substitutions and nucleotide substitutions as described therein.

Table 3.

Designation	Source	AA seq	Nuc. Sequence
E. coli B (reference)	E. coli B	S10; P26; D176; M298;A299;G312; I428	
868PH1	Bison E. coli	I428T	
872PH1	Kangaroo rat E. coli	D176G; G312S M298K; A299T	GAC(176)GGC; GGT(312)AGT; ATG(298)AAG; GCA(299)ACA
875PH2	E. coli W	A160S;D176G; M298K; A299T	GCG(160)TCG; GAC(176)GGC;

		M298K; A299T	ATG(298)AAG; GCA(299)ACA
873PH1	Calf E. coli	I428R	
E. coli B	E. coli B	K298M; T299A	AAG(298)ATG; ACA(299)GCA
K12 appA	E. coli K12	M298K;A299T	ATG(298)AAG; GCA(299)ACA

The enzymes used in the invention can be modified to enhance their activity, delivery, activation and degradation. Such modifications can be performed *in vivo* or *in vitro* and use methods and processes generally known in the art as described more fully below. Such methodology generally uses polynucleotide or polypeptide sequences that are either synthesized by automated machines or are cloned, expressed, or manipulated by recombinant DNA techniques.

In a preferred embodiment, the enzyme used in the compositions (*e.g.*, a dietary aid) of the present invention is a phytase enzyme which is stable to heat and is heat resistant and catalyzes the enzymatic hydrolysis of phytate, *i.e.*, the enzyme is able to renature and regain activity after a brief (*i.e.*, 5 to 30 seconds), or longer period, for example, minutes or hours, exposure to temperatures of above 50 °C.

A "feed" and a "food," respectively, means any natural or artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by an animal and a human being, respectively. "Dietary Aid," as used herein, denotes, for example, a composition containing agents that provide a therapeutic or digestive agent to an animal or organism. A "dietary aid," typically is not a source of caloric intake for an organism, in other words, a dietary aid typically is not a source of energy for the organism, but rather is a composition which is taken in addition to typical "feed" or "food".

An agent or enzyme (*e.g.*, a phytase) may exert its effect *in vitro* or *in vivo*, *i.e.* before intake or in the stomach or gizzard of the organism, respectively. Also a combined action is possible.

Although any enzyme may be incorporated into a dietary aid, reference is made herein to phytase as an exemplification of the methods and compositions of the invention. A dietary aid of the invention includes an enzyme (*e.g.*, a phytase). Generally, a dietary aid containing a phytase composition is liquid or dry.

Liquid compositions need not contain anything more than the enzyme (*e.g.* a phytase), preferably in a highly purified form. Usually, however, a stabilizer such as glycerol, sorbitol or mono propylen glycol is also added. The liquid composition may also comprise other additives, such as salts, sugars, preservatives, pH-adjusting agents, proteins, phytate (a phytase substrate). Typical liquid compositions are aqueous or oil-based slurries. The liquid compositions can be added to a biocompatible composition for slow release. Preferably the enzyme is added to a dietary aid composition that is a biocompatible material (*e.g.*, biodegradable or non-biodegradable) and includes the addition of recombinant cells into, for example, porous microbeads.

Dry compositions may be spray dried compositions, in which case the composition need not contain anything more than the enzyme in a dry form. Usually, however, dry compositions are so-called granulates which may readily be mixed with a food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient means of incorporating enzymes into animal feed. Preferably the granulates are biocompatible and more preferably they biocompatible granulates are non-biodegradable.

Agglomeration granulates coated by an enzyme can be prepared using agglomeration technique in a high shear mixer Absorption granulates are prepared by having cores of a carrier material to absorp/be coated by the enzyme. Preferably the

carrier material is a biocompatible non-biodegradable material that simulates the role of stones or grit in the gizzard of an animal. Typical filler materials used in agglomeration techniques include salts, such as disodium sulphate. Other fillers are kaolin, talc, magnesium aluminium silicate and cellulose fibres. Optionally, binders such as dextrins are also included in agglomeration granulates. The carrier materials can be any biocompatible material including biodegradable and non-biodegradable materials (*e.g.*, rocks, stones, ceramics, various polymers). Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired other additives, such as calcium carbonate or kaolin.

Additionally, the dietary aid compositions (*e.g.*, phytase dietary aid compositions) may contain other substituents such as colouring agents, aroma compounds, stabilizers, vitamins, minerals, other feed or food enhancing enzymes etc. A typical additive usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients.

In a one embodiment, the dietary aid compositions of the invention additionally comprises an effective amount of one or more feed enhancing enzymes, in particular feed enhancing enzymes selected from the group consisting of α-galactosidases, β-galactosidases, in particular lactases, other phytases, β-glucanases, in particular endo-β-1,4-glucanases and endo-β-1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4-β-galactosidases and arabinogalactan endo-1,3-β-galactosidases, endoglucanases, in particular endo-1,2-β-glucanase, endo-1,3-α-glucanase, and endo-1,3-β-glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan-α-rhamnosidase, pectate lyases, and α-galacturonisidases, mannanases, β-mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases.

The animal dietary aid of the invention is supplemented to the mono-gastric animal before or simultaneously with the diet. In one embodiment, the dietary aid of the invention is supplemented to the mono-gastric animal simultaneously with the diet. In another embodiment, the dietary aid is added to the diet in the form of a granulate or a stabilized liquid.

An effective amount of an enzyme in a dietary aid of the invention is from about 10-20,000; preferably from about 10 to 15,000, more preferably from about 10 to 10,000, in particular from about 100 to 5,000, especially from about 100 to about 2,000 FYT/kg dietary aid.

Examples of other specific uses of the phytase of the invention is in soy processing and in the manufacture of inositol or derivatives thereof.

The invention also relates to a method for reducing phytate levels in animal manure, wherein the animal is fed a dietary aid containing an effective amount of the phytase of the invention. As stated in the beginning of the present application one important effect thereof is to reduce the phosphate pollution of the environment.

In another embodiment, the dietary aid is a magnetic carrier. For example, a magnetic carrier containing an enzyme (*e.g.*, a phytase) distributed in, on or through a magnetic carrier (*e.g.*, a porous magnetic bead), can be distributed over an area high in phytate and collected by magnets after a period of time. Such distribution and recollection of beads reduces additional pollution and allows for reuse of the beads. In addition, use of such magnetic beads *in vivo* allows for the localization of the dietary aid to a point in the digestive tract where, for example, phytase activity can be carried out. For example, a dietary aid of the invention containing digestive enzymes (*e.g.*, a phytase) can be localized to the gizzard of the animal by juxtapositioning a magnet next to the gizzard of the animal after the animal consumes a dietary aid of magnetic carriers. The magnet can be removed after a period of time allowing the dietary aid to pass through the digestive tract. In addition, the magnetic carriers are suitable for removal from the organism after sacrificing or to aid in collection.

When the dietary aid is a porous particle, such particles are typically impregnated by a substance with which it is desired to release slowly to form a slow release particle. Such slow release particles may be prepared not only by impregnating the porous particles with the substance it is desired to release, but also by first dissolving the desired substance in the first dispersion phase. In this case, slow release particles prepared by the method in which the substance to be released is first dissolved in the first dispersion phase are also within the scope and spirit of the invention. The porous hollow particles may, for example, be impregnated by a slow release substance such as a medicine, agricultural chemical or enzyme. In particular, when porous hollow particles impregnated by an enzyme are made of a biodegradable polymers, the particles themselves may be used as an agricultural chemical or fertilizer, and they have no adverse effect on the environment. In one embodiment the porous particles are magnetic in nature.

The porous hollow particles may be used as a bioreactor support, in particular an enzyme support. Therefore, it is advantageous to prepare the dietary aid utilizing a method of a slow release, for instance by encapsulating the enzyme of agent in a microvesicle, such as a liposome, from which the dose is released over the course of several days, preferably between about 3 to 20 days. Alternatively, the agent (*e.g.*, an enzyme) can be formulated for slow release, such as incorporation into a slow release polymer from which the dosage of agent (*e.g.*, enzyme) is slowly released over the course of several days, for example from 2 to 30 days and can range up to the life of the animal.

As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multilamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain stabilizers, preservatives, excipients, and the like in addition to the agent. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic.

Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 et seq.

Also within the scope of the invention is the use of a phytase of the invention during the preparation of food or feed preparations or additives, *i.e.*, the phytase excerts its phytase activity during the manufacture only and is not active in the final food or feed product. This aspect is relevant for instance in dough making and baking. Accordingly, phytase or recombinant yeast expressing phytase can be impregnated in, on or through a magnetic carriers, distributed in the dough or food medium, and retrieved by magnets.

The dietary aid of the invention may be administered alone to animals in an biocompatible (*e.g.*, a biodegradable or non-biodegradable) carrier or in combination with other digestion additive agents. The dietary aid of the invention thereof can be readily administered as a top dressing or by mixing them directly into animal feed or provided separate from the feed, by separate oral dosage, by injection or by transdermal means or in combination with other growth related edible compounds, the proportions of each of the compounds in the combination being dependent upon the particular organism or problem being addressed and the degree of response desired. It should be understood that the specific dietary dosage administered in any given case will be adjusted in accordance with the specific compounds being administered, the problem to be treated, the condition of the subject and the other relevant facts that may modify the activity of the effective ingredient or the response of the subject, as is well known by those skilled in the art. In general, either a single daily dose or divided daily dosages may be employed, as is well known in the art.

If administered separately from the animal feed, forms of the dietary aid can be prepared by combining them with non-toxic pharmaceutically acceptable edible carriers to make either immediate release or slow release formulations, as is well known in the art. Such edible carriers may be either solid or liquid such as, for example, corn starch, lactose, sucrose, soy flakes, peanut oil, olive oil, sesame oil and

propylene glycol. If a solid carrier is used the dosage form of the compounds may be tablets, capsules, powders, troches or lozenges or top dressing as micro-dispersable forms. If a liquid carrier is used, soft gelatin capsules, or syrup or liquid suspensions, emulsions or solutions may be the dosage form. The dosage forms may also contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, etc. They may also contain other therapeutically valuable substances.

Thus, a significant advantages of the invention include for example, 1) ease of manufacture of the active ingredient loaded biocompatible compositions; 2) versatility as it relates to the class of polymers and/or active ingredients which may be utilized; 3) higher yields and loading efficiencies; and 4) the provision of sustained release formulations that release active, intact active agents *in vivo*, thus providing for controlled release of an active agent over an extended period of time. In addition, another advantage is due to the local delivery of the agent with in the digestive tract (*e.g.*, the gizzard) of the organism. As used herein the phrase "contained within" denotes a method for formulating an agent into a composition useful for controlled release, over an extended period of time of the agent.

In the sustained-release or slow release compositions of the invention, an effective amount of an agent (*e.g.*, an enzyme or antibiotic) will be utilized. As used herein, sustained release or slow release refers to the gradual release of an agent from a biocompatible material, over an extended period of time. The sustained release can be continuous or discontinuous, linear or non-linear, and this can be accomplished using one or more biodegradable or non-biodegradable compositions, drug loadings, selection of excipients, or other modifications. However, it is to be recognized that it may be desirable to provide for a "fast" release composition, that provides for rapid release once consumed by the organism. It is also to be understood that by "release" does not necessarily mean that the agent is released from the biocompatible carrier. Rather in one embodiment, the slow release encompasses slow activation or continual activation of an agent present on the biocompatible composition. For example, a

phytase need not be released from the biocompatible composition to be effective. In this embodiment, the phytase is immobilized on the biocompatible composition.

The animal feed may be any protein-containing organic meal normally employed to meet the dietary requirements of animals. Many of such protein-containing meals are typically primarily composed of corn, soybean meal or a corn/soybean meal mix. For example, typical commercially available products fed to fowl include Egg Maker Complete, a poultry feed product of Land O'Lakes AG Services, as well as Country Game & Turkey Grower a product of Agwa, Inc. (see also The Emu Farmer's Handbook by Phillip Minnaar and Maria Minnaar). Both of these commercially available products are typical examples of animal feeds with which the present dietary aid and/or the enzyme phytase may be incorporated to reduce or eliminate the amount of supplemental phosphorus, zinc, manganese and iron intake required in such compositions.

The present invention is applicable to the diet of numerous animals, which herein is defined as including mammals (including humans), fowl and fish. In particular, the diet may be employed with commercially significant mammals such as pigs, cattle, sheep, goats, laboratory rodents (rats, mice, hamsters and gerbils), fur-bearing animals such as mink and fox, and zoo animals such as monkeys and apes, as well as domestic mammals such as cats and dogs. Typical commercially significant avian species include chickens, turkeys, ducks, geese, pheasants, emu, ostrich, loons, kiwi, doves, parrots, cockatiel, cockatoo, canaries, penguins, flamingoes, and quail. Commercially farmed fish such as trout would also benefit from the dietary aids disclosed herein. Other fish that can benefit include, for example, fish (especially in an aquarium or acquaculture environment, *e.g.*, tropical fish), goldfish and other ornamental carp, catfish, trout, salmon, shark, ray, flounder, sole, tilapia, medaka, guppy, molly, platyfish, swordtail, zebrafish, and loach.

Unless otherwise stated, transformation was performed as described in the method of Sambrook, Fritsch and Maniatus, 1989. The following examples are intended to illustrate, but not to limit, the invention. While the procedures described

in the examples are typical of those that can be used to carry out certain aspects of the invention, other procedures known to those skilled in the art can also be used.

EXAMPLE 1

ISOLATION, BACTERIAL EXPRESSION, AND PURIFICATION OF PHYTASE

E.coli B genomic DNA was obtained from Sigma (Catalog # D-2001), St. Louis, New Jersey.

The following primers were used to PCR amplify the gene directly from the genomic DNA:

5' primer gtttctgaattcaaggaggaatttaaATGAAAGCGATCTTAATCCCATT (SEQ ID NO:3); and

3' primer gtttctggatccTTACAAACTGCACGCCGGTAT (SEQ ID NO:4).

Pfu polymerase in the PCR reaction, and amplification was performed according to manufacturers protocol (Stratagene Cloning Systems, Inc., La Jolla, CA).

PCR product was purified and purified product and pQE60 vector (Qiagen) were both digested with EcoRI and BglII restriction endonucleases (New England Biolabs) according to manufacturers protocols. Overnight ligations were performed using standard protocols to yield pQE60.

The amplified sequences were inserted in frame with the sequence encoding for the RBS. The ligation mixture was then used to transform the *E. coli* strain M15/pREP4 (Qiagen, Inc.) by electroporation. M15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N

culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 ($O.D.^{600}$) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation.

The primer sequences set out above may also be employed to isolate the target gene from the deposited material by hybridization techniques described above.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described. It is to be understood that, while the invention has been described with reference to the above detailed description, the foregoing description is intended to illustrate, but not to limit, the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the following claims. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

This invention also provides for the isolation and use of phytase molecules (nucleic acids and phytase enzymes encoded thereby) from all other strains of E. coli (whether virulent or non-virulent, including K12, W, C), as well as all bacteria. These include all known species and strains belonging to:

Thermotogales
Green Nonsulfur Bacteria
Cyanobacteria & chloroplasts
Low G+C Gram-Positive Bacteria
Fusobacteria
High G+C Gram-Positive Bacteria
Gytophaga/Flexibacter/Bacteroides group
Fibrobacteria
Spriochaetes
Planctomyces/Chlamydia group

Purple bacteria (Proteobacteria), including the following subdivisions:
- Delta & Epsilon, including:
  - Desulfuromonas acetoxidans
  - Desulfosarcina variabilis
  - Bdellovibrio stolpii
  - Nannocystis exedens
  - Stigmatella aurantiaca
  - Myxococcus xanthus
  - Desulfovibrio desulfuricans
  - Thiovulum sp.
  - Campylobacter jejuni
  - Wolinella succinogenes
  - Helicobacter pylori
- Alpha, including:
  - Methylobacterium extorquens
  - Beijerinckia indica
  - Hyphomicrobium vulgare
  - Rhodomicrobium vannieli
  - Agrobacterium tumefaciens
  - Brucella abortus
  - Rochalimaea quintana
  - Rhodopseudomonas marina subsp. agilis
  - Zea mays – mitochondrion
  - Rickettsia rickettsii
  - Ehrlichia risticii
  - Wolbachia pipientis
  - Anaplasma marginale
  - Erythrobacter longus
  - Rhodospirillum salexigens
  - Rhodobacter capsulatus
  - Azospirillum lipoferum
  - Rhodospirillum rubrum
- Gamma, including:
  - Ectothiorhodospira shaposhnikovii
  - Chromatium vinosum
  - Methylomonas methanica
  - Cardiobacterium hominis
  - Xanthomonas maltophilia
  - Coxiella burnetii
  - Legionella pneumophila subsp. pneumophila
  - Oceanospirillum linum
  - Acinetobacter calcoaceticus
  - Pseudomonas aeruginosa
  - Haemophilus influenzae
  - Vibrio parahaemolyticus

Proteus vulgaris
Erwinia carotovora
Echerichia coli, including:
Beta, including:
Eikenella corrodens
Neisseria gonorrhoeae
Vitreoscilla stercoraria
Chromobacterium violaceum
Alcaligenes faecalis
Rubrivivax gelatinosus
Pseudomonas testosteroni
Nitrosomonas europae
Spirillum volutans

Such phytase molecules can be isolated from these bacteria by know methods, including library screening methods, *e.g.* expression screening, hybridization methods, PCR (*e.g.* see Sammbrook, 1989).

EXAMPLE 1

THERMAL TOLERANCE ASSAY

The wild type appA from E. coli (strain K12) and a mutagenized version designated 819PH59 (SEQ ID NO:9 and 10) were expressed in E. coli and purified to homogeneity. In the thermal tolerance assay, 100 uL of 0.01 mg/mL of protein in 100 mM MOPS / pH 7.0 was heated to the indicated incubation temperature for 5 minutes in an RJ research thermocycler. Upon completion of the 5 minutes at temperature, the samples were cooled to 4°C and incubated on ice. An activity assay was run using 40 uL of the enzyme solution in 1.5 mL of 100 mM NaOAc / 4 mM phytate / pH 4.5 at 37 °C. Aliquots of 60 uL were withdrawn at 2 minute intervals and added to 60 uL of the color developer/Stop solution of the TNO assay. Clearly, the modified enzyme, SEQ ID NO:10, containing 8 amino acid changes, is tolerant to temperatures greater than the wild-type enzyme. (see Figure 3)

EXAMPLE 2

STABILITY OF PHYTASE ENZYME IN SIMULATED DIGESTIBILITY CONDITIONS

The percent residual activities (based on initial rates) of the in vitro digested E.coli K12 and the nonglycosylated 819pH59 phytase were plotted verses time. A standard concentration of simulated gastric fluid containing 2 mg/ml NaCl, 6 M HCl and 3.2 mg/mL pepsin was prepared as described. The pH of the solution was about 1.4 and was not adjusted. The in vitro digestibility assay was performed by adding 1:4 (vol:vol) of phytase to digestion solution and immediately incubating at 37 o C to initiate the digestion reaction. Aliquots of the digestion reaction mixture were removed at various time intervals and assayed for residual phytase activity using the TNO assay. Each of the assays was performed at least twice. An exponential curve with the equation $y = Ae^{-kt}$ was fit to the data. The half lives of the proteins were determined using the equation $t\ 1/2 = \ln 2/ k$. The half-life of the E.coli K12 phytase was only 2.7 ± 0.2 minutes while the nonglycosylated 819pH59 phytase had a half-life of 8.4 ± 1.1 minutes. Therefore, the mutations in the wildtype E.coli K12 phytase enhanced the stability of the enzyme under simulated in vitro digestibility conditions.

See Figure 4.

EXAMPLE 3

EXPRESSION HOST COMPARISONS

The GSSM DNA construct from 819PH59 was inserted into E. coli, P. pastoris, and S. pombe for expression. The expressed proteins were purified to homogeneity. In the thermal tolerance assay, 100 uL of 0.01 mg/mL of protein in 100 mM MOPS, pH 7.0 was heated to the indicated incubation temperature for 5 minutes in an RJ research thermocycler. Upon completion of the 5 minutes at temperature, the samples were cooled to 4°C and incubated on ice. An activity assay was run using 40 uL of the enzyme solution in 1.46 mL of 100 mM NaOAc / 4 mM phytate / pH 4.5 at

37 °C. Aliquots of 60 uL were withdrawn at 2 minute intervals and added to 60 uL of the color developer/Stop solution of the TNO assay. (see Figure 5).

EXAMPLE 4

The percent residual activities (based on initial rates) of the *in vitro* digested 819pH59 phytase expressed in various expression hosts were plotted verses time. The 819pH59 phytase was expressed in *E.coli* (nonglycosylated), as well as in *S.pombe* and *P. pastoris* (glycosylated). A standard concentration of simulated gastric fluid containing 2 mg/ml NaCl, 6 M HCl and 3.2 mg/mL pepsin was prepared as described in the S.O.P. The pH of the solution was about 1.4 and was not adjusted. The *in vitro* digestibility assay was performed by adding 1:4 (vol:vol) of phytase to digestion solution and immediately incubating at 37 °C to initiate the digestion reaction. Aliquots of the digestion reaction mixture were removed at various time intervals and assayed for residual phytase activity using the TNO assay. Each of the assays was performed in triplicate. An exponential curve with the equation $y = Ae^{-kt}$ was fit to the data. The half lives of the proteins were determined using the equation $t_{1/2} = \ln 2/k$. The half-life of the nonglycosylated 819pH59 phytase expressed in *E.coli* was 8.4 ± 1.1 minutes while the glycosylated 819pH59 phytase expressed in *S. pombe* had a half-life of 10.4 ± 0.9 minutes and the same phytase expressed in *P. pastoris* had a half-life of 29.2 ± 6.7 mins. Therefore, the glycosylation of the 819pH59 phytase enhanced the stability of the enzyme under simulated *in vitro* digestibility conditions. (see figure 6)

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(The teachings of all references cited in this application are hereby incorporated by reference in their entirety unless otherwise indicated.)

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What is claimed is:

1. A method for improving the nutritional value of a phytate-containing foodstuff comprising:

contacting said phytate-containing foodstuff with a substantially pure phytase enzyme having an amino acid sequence of SEQ ID NO:2, such that said substantially pure phytase enzyme catalyzes the liberation of inorganic phosphate from the phytate in said phytate-containing foodstuff.

2. The method of claim 1, wherein said substantially pure phytase enzyme is produced by a recombinant expression system comprising a phytase-encoding nucleic acid having a nucleotide sequence selected from the group consisting of:

a) SEQ ID NO:1, and

b) SEQ ID NO:1 wherein T can also be U;

wherein the expression of the phytase-encoding nucleic acid leads to the production of said substantially pure phytase enzyme.

3. The method of claim 1, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs prior to the ingestion of said phytate-containing foodstuff by a recipient organism.

4. The method of claim 1, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs after the ingestion of said phytate-containing foodstuff by a recipient organism.

5. The method of claim 1, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs in part prior to and in part after the ingestion of said phytate-containing foodstuff by a recipient organism.

6. A recombinant expression system for use in the method of claim 2, wherein said recombinant expression system is contained in a host cell and expresses a nucleotide sequence encoding a phytase enzyme having an amino acid sequence as set forth in SEQ ID NO:2, and wherein the nucleotide sequence encoding said enzyme is operably linked to a transcription control sequence operable in said host cell.

7. A vector which comprises the expression system according to claim 6.

8. The expression system of claim 6, wherein the control sequence comprises a constitutive promoter.

9. The expression system of claim 6, wherein the control sequence comprises a tissue-specific promoter.

10. The expression system of claim 6, wherein said host cell is a prokaryotic cell.

11. The expression system of claim 6, wherein said host cell is a eukaryotic cell.

12. The expression system of claim 6, wherein said host cell is a plant cell.

13. The expression system of claim 6, wherein said nucleotide sequence is preceded by a polynucleotide sequence encoding a signal peptide operably linked to said nucleotide sequence.

14. The expression system of claim 13, wherein said signal peptide is the PR protein PR-S signal peptide from tobacco.

15. A prokaryotic cell modified to contain the expression system of claim 6.

16. A eukaryotic cell modified to contain the expression system of claim 6.

17. A plant cell, plant part or plant modified to contain the expression system of claim 6.

18. A method to produce an animal feed containing a microbial phytase comprising:

a) culturing the plant cell, plant part or plant of claim 17 under conditions wherein said nucleotide sequence is expressed; and

b) converting said plant cells, plant parts or plants into a composition suitable for animal feed.

19. A feed composition for animals which comprises the plant seeds, plant cells, plant parts or plants of claim 17 in admixture with a phytate-containing foodstuff.

20. A method to treat a human or an animal able to benefit from digestive enhancement by the activity of an exogenous phytase enzyme, which method comprises administering to said human or animal an amount of plant seeds, plant cells, plant parts or plants of a transgenic plant, wherein said transgenic plant is modified to contain an expression system which expresses a nucleotide sequence encoding a phytase enzyme in an amount effective to provide phytase activity in said human's or animal's digestive tract.

21. A transgenic non-human organism whose genome comprises a heterologous nucleic acid sequence encoding a polypeptide having phytase activity, wherein said transgene results in expression of a phytase polypeptide.

22. A method of generating a variant comprising:

obtaining a nucleic acid comprising a sequence as set forth in SEQ ID NO: 1, sequences substantially identical thereto, sequences complementary thereto, fragments comprising at least 30 consecutive nucleotides thereof, and fragments comprising at least 30 consecutive nucleotides of the sequences complementary to SEQ ID NO:1; and

modifying one or more nucleotides in said sequence to another nucleotide, deleting one or more nucleotides in said sequence, or adding one or more nucleotides to said sequence.

23. The method of claim 22, wherein the modifications are introduced by a method selected from the group consisting of error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, ligation reassembly, GSSM and any combination thereof.

24. The method of claim 22, wherein the modifications are introduced by error-prone PCR.

25. The method of claim 22, wherein the modifications are introduced by shuffling.

26. The method of claim 22, wherein the modifications are introduced by oligonucleotide-directed mutagenesis.

27. The method of claim 22, wherein the modifications are introduced by assembly PCR.

28. The method of claim 22, wherein the modifications are introduced by sexual PCR mutagenesis.

29. The method of claim 22, wherein the modifications are introduced by *in vivo* mutagenesis.

30. The method of claim 22, wherein the modifications are introduced by cassette mutagenesis.

31. The method of claim 22, wherein the modifications are introduced by recursive ensemble mutagenesis.

32. The method of claim 22, wherein the modifications are introduced by exponential ensemble mutagenesis.

33. The method of claim 22, wherein the modifications are introduced by site-specific mutagenesis.

34. A computer readable medium having stored thereon a nucleic acid sequence as set forth in SEQ ID NO:1, and sequences substantially identical thereto, or a polypeptide sequence as set forth in SEQ ID NO:2, and sequences substantially identical thereto.

35. A computer system comprising a processor and a data storage device wherein said data storage device has stored thereon a nucleic acid sequence as set forth in SEQ ID NO:1, and sequences substantially identical thereto, or a polypeptide sequence as set forth in SEQ ID NO:2 and sequences substantially identical thereto.

36. The computer system of claim 35, further comprising a sequence comparison algorithm and a data storage device having at least one reference sequence stored thereon.

37. The computer system of claim 36, wherein the sequence comparison algorithm comprises a computer program which indicates polymorphisms.

38. The computer system of claim 35, further comprising an identifier which identifies features in said sequence.

39. A method for comparing a first sequence to a reference sequence or a database of sequences wherein said first sequence is a nucleic acid sequence as set forth in SEQ ID NO:1, and sequences substantially identical thereto, or a polypeptide sequence as set forth in SEQ ID NO:2, and sequences substantially identical thereto comprising:

reading the first sequence and the reference sequence or database of sequences through use of a computer program which compares sequences; and

determining differences between the first sequence and the reference sequence or database of sequences with the computer program.

40. The method of claim 39, wherein determining differences between the first sequence and the reference sequence comprises identifying polymorphisms.

41. An isolated polynucleotide encoding a phytase enzyme having an amino acid sequence as set forth in SEQ ID NO:8, and having one or more amino acid modifications selected from W68E, Q84W, A95P, K97C, S168E, R180Y, N226C, Y277D or any combination thereof.

42. An isolated polynucleotide, or an oligonucleotide portion thereof, comprising a contiguous sequence of at least about ten nucleotides substantially identical to a nucleotide sequence of SEQ ID NO:7 or to a nucleotide sequence complementary thereto, the contiguous nucleotide sequence comprising nucleotide changes wherein at least nucleotide 390 is G; 391 is A; nucleotide 438 is T; 439 is G; 440 is G; 471 is C; 473 is T; 477 is T; 448 is G; 449 is T; 690 is G; 691 is A; 692 is G; 729 is T; 730 is A; 731 is T; 864 is T; 865 is G; 1017 is G or any combination thereof.

43. The polynucleotide of claim 41, wherein the polynucleotide has a nucleotide sequence 90% homologous to a sequence as set forth in SEQ ID NO:9.

44. The polynucleotide of claim 41, wherein the polynucleotide has a nucleotide sequence as set forth in SEQ ID NO:9.

45. An isolated polynucleotide comprising a nucleotide sequence substantially identical to SEQ ID NO:7, and wherein nucleotide 390 is G; 391 is A; nucleotide 438 is T; 439 is G; 440 is G; 471 is C; 473 is T; 477 is T; 448 is G; 449 is T; 690 is G; 691 is A; 692 is G; 729 is T; 730 is A; 731 is T; 864 is T; 865 is G; 1017 is G, or any combination thereof.

46. The polynucleotide of claim 45, comprising a nucleotide sequence substantially identical to SEQ ID NO:7, and having a modified nucleotide sequence selected from nucleotide 390 is G and 391 is A; nucleotide 438 is T, 439 is G and 440 is G; 471 is C and 473 is T; 477 is T, 448 is G, and 449 is T; 690 is G, 691 is A and 692 is G; 729 is T, 730 is A, and 731 is T; 864 is T and 865 is G; 1017 is G, or any combination thereof.

47. An expression vector containing a polynucleotide of any of claims 41,4 4, or 46.

48. The vector of claim 47, wherein the vector is a viral vector.

49. The vector of claim 47, wherein the vector is a bacterial vector.

50. A host cell containing a vector of claim 47.

51. The host cell of claim 50, wherein the cell is a prokaryotic cell.

52. The host cell of claim 51, wherein the cell is E. coli, P. pastoris, or S. pombe.

53. The host cell of claim 50, wherein the cell is a eukaryotic cell.

54. A substantially purified polypeptide having an amino acid sequence substantially identical to SEQ ID NO:8 and having one or more amino acid modifications selected from W68E, Q84W, A95P, K97C, S168E, R180Y, N226C, Y277D or any combination thereof, wherein the polypeptide has phytase activity.

55. The polypeptide of claim 54, wherein the polypeptide activity is tolerant to temperatures of at least 60 degrees C.

56. The polypeptide of claim 54, wherein the polypeptide activity is tolerant to temperatures of at least 70 degrees C.

57. The polypeptide of claim 54, wherein the polypeptide activity is tolerant to temperatures of at least 80 degrees C.

58. The polypeptide of claim 54, wherein the polypeptide has a half-life in gastric fluid at least about 2-fold greater than the corresponding wild-type polypeptide.

59. A substantially purified polypeptide having an amino acid sequence as set forth in SEQ ID NO:10.

60. A method for improving the nutritional value of a phytate-containing foodstuff comprising:

contacting said phytate-containing foodstuff with a substantially pure phytase enzyme having an amino acid sequence of a polypeptide of claim 15, such that said substantially pure phytase enzyme catalyzes the liberation of inorganic phosphate from the phytate in said phytate-containing foodstuff.

61. The method of claim 60, wherein said substantially pure phytase enzyme is produced by a recombinant expression system comprising a phytase-encoding nucleic acid having a nucleotide sequence selected from the group consisting of:

a) SEQ ID NO:9;

b) SEQ ID NO:9 wherein T can also be U;

wherein the expression of the phytase-encoding nucleic acid leads to the production of said substantially pure phytase enzyme; and

c) SEQ ID NO:7, wherein 390 is G; 391 is A; nucleotide 438 is T; 439 is G; 440 is G; 471 is C; 473 is T; 477 is T; 448 is G; 449 is T; 690 is G; 691 is A; 692 is G; 729 is T; 730 is A; 731 is T; 864 is T; 865 is G; 1017 is G, or any combination thereof.

62. The method of claim 60, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs prior to the ingestion of said phytate-containing foodstuff by a recipient organism.

63. The method of claim 60, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs after the ingestion of said phytate-containing foodstuff by a recipient organism.

64. The method of claim 60, wherein the liberation of the inorganic phosphate from the phytate in said phytate-containing foodstuff occurs in part prior to and in part after the ingestion of said phytate-containing foodstuff by a recipient organism.

65. A recombinant expression system for use in the method of claim 61, wherein said recombinant expression system is contained in a host cell and expresses a nucleotide sequence encoding a phytase enzyme having an amino acid sequence as set forth in SEQ ID NO:9, and wherein the nucleotide sequence encoding said enzyme is operably linked to a transcription control sequence operable in said host cell.

66. A vector which comprises the expression system according to claim 65.

67. The expression system of claim 65, wherein the control sequence comprises a constitutive promoter.

68. The expression system of claim 65, wherein the control sequence comprises a tissue-specific promoter.

69. The expression system of claim 65, wherein said host cell is a prokaryotic cell.

70. The expression system of claim 65, wherein said host cell is a eukaryotic cell.

71. The expression system of claim 65, wherein said host cell is a plant cell.

72. The expression system of claim 65, wherein said nucleotide sequence is preceded by a polynucleotide sequence encoding a signal peptide operably linked to said nucleotide sequence.

73. The expression system of claim 72, wherein said signal peptide is the PR protein PR-S signal peptide from tobacco.

74. A prokaryotic cell modified to contain the expression system of claim 65.

75. A eukaryotic cell modified to contain the expression system of claim 65.

76. A plant cell, plant part or plant modified to contain the expression system of claim 65.

77. A method to produce an animal feed containing a microbial phytase comprising:

a) culturing the plant cell, plant part or plant of claim 76 under conditions wherein said nucleotide sequence is expressed; and

b) converting said plant cells, plant parts or plants into a composition suitable for animal feed.

78. A feed composition for animals which comprises the plant seeds, plant cells, plant parts or plants of claim 76 in admixture with a phytate-containing foodstuff.

79. A method of generating a variant comprising:

obtaining a nucleic acid comprising a sequence as set forth in SEQ ID NO:9, sequences substantially identical thereto, sequences complementary thereto, fragments comprising at least 30 consecutive nucleotides thereof, and fragments comprising at least 30 consecutive nucleotides of the sequences complementary to SEQ ID NO:9; and

modifying one or more nucleotides in said sequence to another nucleotide, deleting one or more nucleotides in said sequence, or adding one or more nucleotides to said sequence.

80. The method of claim 79, wherein the modifications are introduced by a method selected from the group consisting of error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, ligation reassembly, GSSM and any combination thereof.

81. The method of claim 79, wherein the modifications are introduced by error-prone PCR.

82. The method of claim 79, wherein the modifications are introduced by shuffling.

83. The method of claim 79, wherein the modifications are introduced by oligonucleotide-directed mutagenesis.

84. The method of claim 79, wherein the modifications are introduced by assembly PCR.

85. The method of claim 79, wherein the modifications are introduced by sexual PCR mutagenesis.

86. The method of claim 79, wherein the modifications are introduced by *in vivo* mutagenesis.

87. The method of claim 79, wherein the modifications are introduced by cassette mutagenesis.

88. The method of claim 79, wherein the modifications are introduced by recursive ensemble mutagenesis.

89. The method of claim 79, wherein the modifications are introduced by exponential ensemble mutagenesis.

90. The method of claim 79, wherein the modifications are introduced by site-specific mutagenesis.

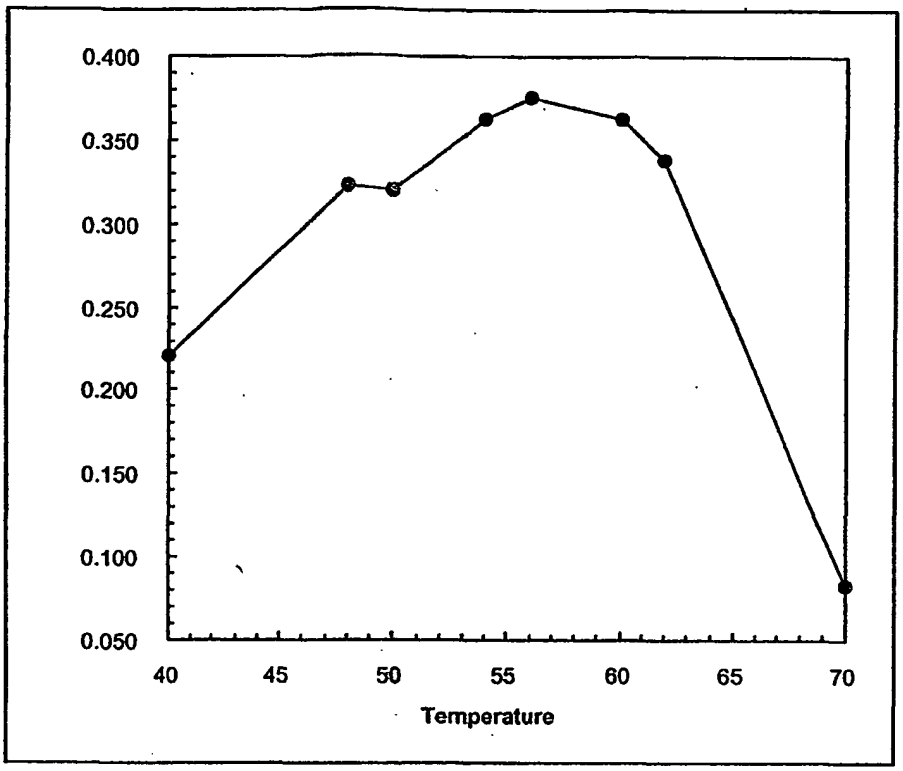
FIGURE 1

(SEQ ID NO:1-nucleotide sequence and SEQ ID NO:2-amino acid sequence)
***Escherichia coli* B Phytase Sequence**

```
1
ATG AAA GCG ATC TTA ATC CCA TTT TTA TCT CTT CTG ATT CCG TTA ACC CCG
Met Lys Ala Ile Leu Ile Pro Phe Leu Ser Leu Leu Ile Pro Leu Thr Pro

CAA TCT GCA TTC GCT CAG AGT GAG CCG GAG CTG AAG CTG GAA AGT GTG GTG
Gln Ser Ala Phe Ala Gln Ser Glu Pro Glu Leu Lys Leu Glu Ser Val Val

ATT GTC AGT CGT CAT GGT GTG CGT GCT CCA ACC AAG GCC ACG CAA CTG ATG
Ile Val Ser Arg His Gly Val Arg Ala Pro Thr Lys Ala Thr Gln Leu Met

CAG GAT GTC ACC CCA GAC GCA TGG CCA ACC TGG CCG GTA AAA CTG GGT TGG
Gln Asp Val Thr Pro Asp Ala Trp Pro Thr Trp Pro Val Lys Leu Gly Trp

CTG ACA CCG CGN GGT GGT GAG CTA ATC GCC TAT CTC GGA CAT TAC CAA CGC
Leu Thr Pro Arg Gly Gly Glu Leu Ile Ala Tyr Leu Gly His Tyr Gln Arg

CAG CGT CTG GTA GCC GAC GGA TTG CTG GCG AAA AAG GGC TGC CCG CAG TCT
Gln Arg Leu Val Ala Asp Gly Leu Leu Ala Lys Lys Gly Cys Pro Gln Ser

GGT CAG GTC GCG ATT ATT GCT GAT GTC GAC GAG CGT ACC CGT AAA ACA GGC
Gly Gln Val Ala Ile Ile Ala Asp Val Asp Glu Arg Thr Arg Lys Thr Gly

GAA GCC TTC GCC GCC GGG CTG GCA CCT GAC TGT GCA ATA ACC GTA CAT ACC
Glu Ala Phe Ala Ala Gly Leu Ala Pro Asp Cys Ala Ile Thr Val His Thr

CAG GCA GAT ACG TCC AGT CCC GAT CCG TTA TTT AAT CCT CTA AAA ACT GGC
Gln Ala Asp Thr Ser Ser Pro Asp Pro Leu Phe Asn Pro Leu Lys Thr Gly

GTT TGC CAA CTG GAT AAC GCG AAC GTG ACT GAC GCG ATC CTC AGC AGG GCA
Val Cys Gln Leu Asp Asn Ala Asn Val Thr Asp Ala Ile Leu Ser Arg Ala

GGA GGG TCA ATT GCT GAC TTT ACC GGG CAT CGG CAA ACG GCG TTT CGC GAA
Gly Gly Ser Ile Ala Asp Phe Thr Gly His Arg Gln Thr Ala Phe Arg Glu

CTG GAA CGG GTG CTT AAT TTT CCG CAA TCA AAC TTG TGC CTT AAA CGT GAG
Leu Glu Arg Val Leu Asn Phe Pro Gln Ser Asn Leu Cys Leu Lys Arg Glu

AAA CAG GAC GAA AGC TGT TCA TTA ACG CAG GCA TTA CCA TCG GAA CTC AAG
Lys Gln Asp Glu Ser Cys Ser Leu Thr Gln Ala Leu Pro Ser Glu Leu Lys

GTG AGC GCC GAC AAT GTC TCA TTA ACC GGT GCG GTA AGC CTC GCA TCA ATG
Val Ser Ala Asp Asn Val Ser Leu Thr Gly Ala Val Ser Leu Ala Ser Met

CTG ACG GAG ATA TTT CTC CTG CAA CAA GCA CAG GGA ATG CCG GAG CCG GGG
Leu Thr Glu Ile Phe Leu Leu Gln Gln Ala Gln Gly Met Pro Glu Pro Gly

TGG GGA AGG ATC ACC GAT TCA CAC CAG TGG AAC ACC TTG CTA AGT TTG CAT
Trp Gly Arg Ile Thr Asp Ser His Gln Trp Asn Thr Leu Leu Ser Leu His

AAC GCG CAA TTT TAT TTG CTA CAA CGC ACG CCA GAG GTT GCC CGC AGC CGC
Asn Ala Gln Phe Tyr Leu Leu Gln Arg Thr Pro Glu Val Ala Arg Ser Arg
```

```
GCC ACC CCG TTA TTG GAT TTG ATC ATG GCA GCG TTG ACG CCC CAT CCA CCG
Ala Thr Pro Leu Leu Asp Leu Ile Met Ala Ala Leu Thr Pro His Pro Pro

CAA AAA CAG GCG TAT GGT GTG ACA TTA CCC ACT TCA GTA CTG TTT ATT GCC
Gln Lys Gln Ala Tyr Gly Val Thr Leu Pro Thr Ser Val Leu Phe Ile Ala

GGA CAC GAT ACT AAT CTG GCA AAT CTC GGC GGC GCA CTG GAG CTC AAC TGG
Gly His Asp Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu Leu Asn Trp

ACG CTT CCC GGT CAG CCG GAT AAC ACG CCG CCA GGT GGT GAA CTG GTG TTT
Thr Leu Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly Glu Leu Val Phe

GAA CGC TGG CGT CGG CTA AGC GAT AAC AGC CAG TGG ATT CAG GTT TCG CTG
Glu Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp Ile Gln Val Ser Leu

GTC TTC CAG ACT TTA CAG CAG ATG CGT GAT AAA ACG CCG CTG TCA TTA AAT
Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys Thr Pro Leu Ser Leu Asn

ACG CCG CCC GGA GAG GTG AAA CTG ACC CTG GCA GGA TGT GAA GAG CGA AAT
Thr Pro Pro Gly Glu Val Lys Leu Thr Leu Ala Gly Cys Glu Glu Arg Asn

GCG CAG GGC ATG TGT TCG TTG GCA GGT TTT ACG CAA ATC GTG AAT GAA GCA
Ala Gln Gly Met Cys Ser Leu Ala Gly Phe Thr Gln Ile Val Asn Glu Ala

CGC ATA CCG GCG TGC AGT TTG AGA TCT CAT CAC CAT CAC CAT CAC TAA  1323
Arg Ile Pro Ala Cys Ser Leu Arg Ser His His His His His His End
```

FIGURE 2
pH/Temperature Profile and Stability



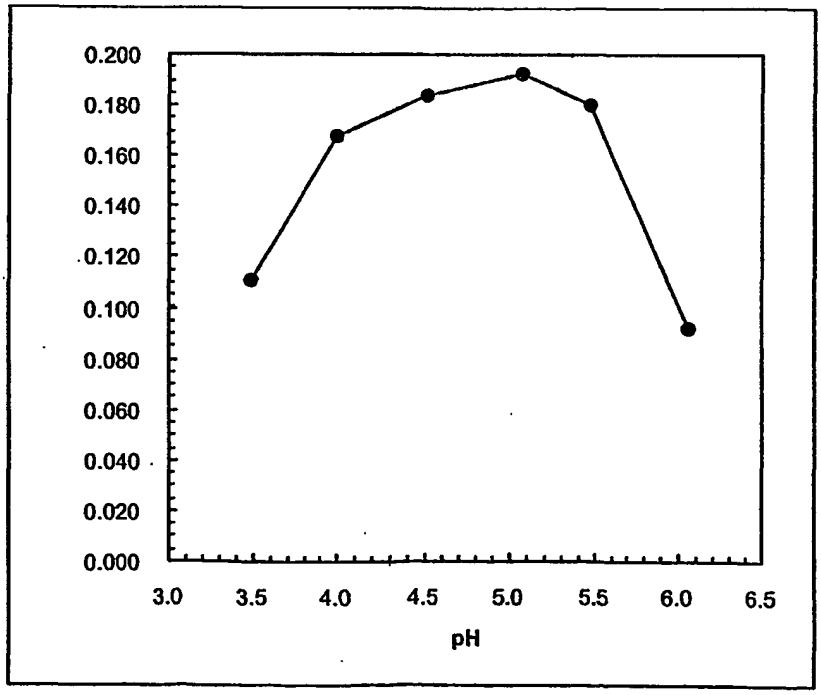


FIGURE 3

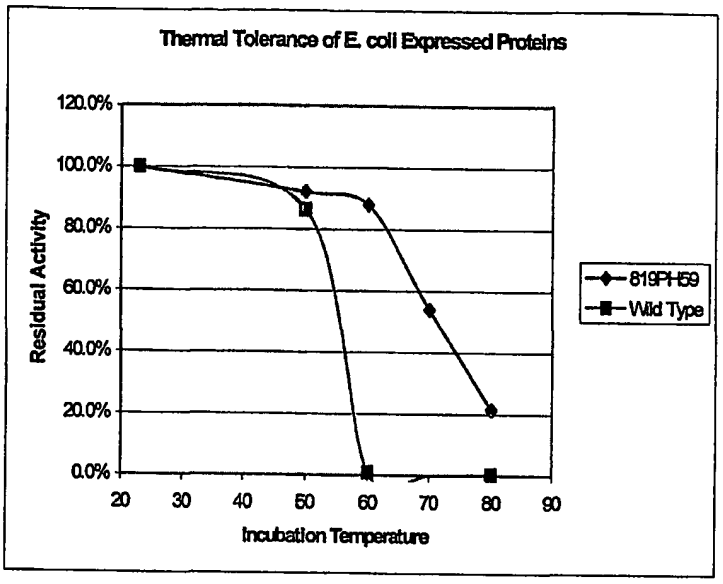

Thermal Tolerance of E. coli Expressed Proteins
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Residual Activity
20
30
40
50
60
70
80
90
Incubation Temperature
819PH59
Wild Type

FIGURE 4

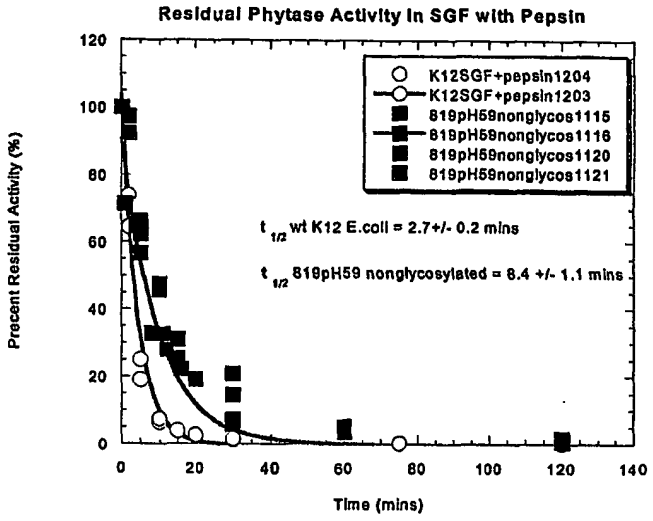

Residual Phytase Activity in SGF with Pepsin
Precent Residual Activity (%)
Time (mins)
K12SGF+pepsin1204
K12SGF+pepsin1203
819pH59nonglycos1115
819pH59nonglycos1116
819pH59nonglycos1120
819pH59nonglycos1121
t 1/2 wt K12 E.coli = 2.7+/- 0.2 mins
t 1/2 819pH59 nonglycosylated = 8.4 +/- 1.1 mins
0
20
40
60
80
100
120
140

FIGURE 5

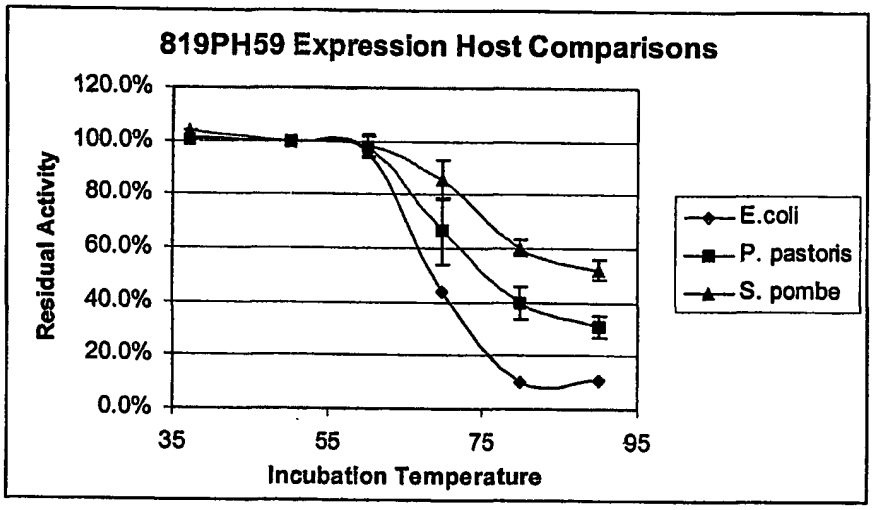

819PH59 Expression Host Comparisons
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Residual Activity
35
55
75
95
Incubation Temperature
E.coli
P. pastoris
S. pombe

FIGURE 6

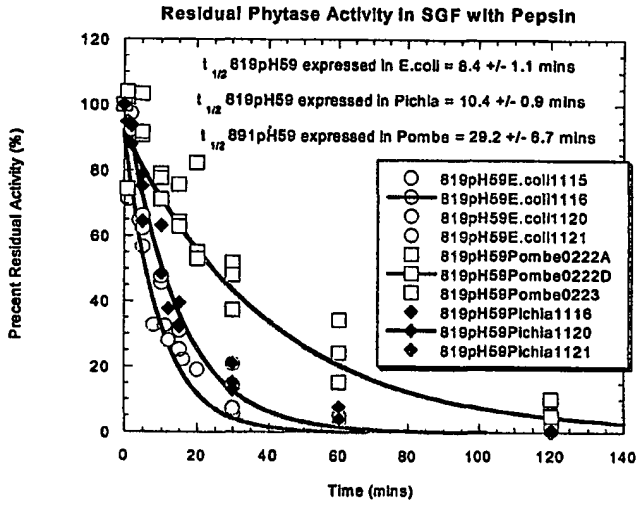

Residual Phytase Activity in SGF with Pepsin
t 1/2 819pH59 expressed in E.coli = 8.4 +/- 1.1 mins
t 1/2 819pH59 expressed in Pichia = 10.4 +/- 0.9 mins
t 1/2 891pH59 expressed in Pombe = 29.2 +/- 6.7 mins
819pH59E.coli1115
819pH59E.coli1116
819pH59E.coli1120
819pH59E.coli1121
819pH59Pombe0222A
819pH59Pombe0222D
819pH59Pombe0223
819pH59Pichia1116
819pH59Pichia1120
819pH59Pichia1121
Precent Residual Activity (%)
Time (mins)
0
20
40
60
80
100
120
140

FIGURE 7A

E. coli appA (GenBank accession no. M58708) (SEQ ID NO:7)

1 taaggagcag aaacaatgtg gtatttactt tggttcgtcg gcattttgtt gatgtgttcg
61 ctctccaccc ttgtgttggt atggctggac ccgcgtctga aaagttaacg aacgtaggcc
121 tgatgcggcg cattagcatc gcatcaggca atcaataatg tcagatatga aaagcggaaa
181 catatcgatg aaagcgatct taatcccatt tttatctctt ctgattccgt taaccccgca
241 atctgcattc gctcagagtg agccggagct gaagctggaa agtgtggtga ttgtcagtcg
301 tcatggtgtg cgtgctccaa ccaaggccac gcaactgatg caggatgtca ccccagacgc
361 atggccaacc tggccggtaa aactgggttg gctgacaccg cgnggtggtg agctaatcgc
421 ctatctcgga cattaccaac gccagcgtct ggtagccgac ggattgctgg cgaaaaaggg
481 ctgcccgcag tctggtcagg tcgcgattat tgctgatgtc gacgagcgta cccgtaaaac
541 aggcgaagcc ttcgccgccg ggctggcacc tgactgtgca ataaccgtac atacccaggc
601 agatacgtcc agtcccgatc cgttatttaa tcctctaaaa actggcgttt gccaactgga
661 taacgcgaac gtgactgacg cgatcctcag cagggcagga gggtcaattg ctgactttac
721 cgggcatcgg caaacggcgt ttcgcgaact ggaacgggtg cttaattttc cgcaatcaaa
781 cttgtgcctt aaacgtgaga aacaggacga aagctgttca ttaacgcagg cattaccatc
841 ggaactcaag gtgagcgccg acaatgtctc attaaccggt gcggtaagcc tcgcatcaat
901 gctgacggag atatttctcc tgcaacaagc acagggaatg ccggagccgg ggtggggaag
961 gatcaccgat tcacaccagt ggaacacctt gctaagtttg cataacgcgc aattttattt
1021 gctacaacgc acgccagagg ttgcccgcag ccgcgccacc ccgttattag atttgatcaa
1081 gacagcgttg acgccccatc caccgcaaaa acaggcgtat ggtgtgacat tacccacttc
1141 agtgctgttt atcgccggac acgatactaa tctggcaaat ctcggcggcg cactggagct
1201 caactggacg cttcccggtc agccggataa cacgccgcca ggtggtgaac tggtgtttga
1261 acgctggcgt cggctaagcg ataacagcca gtggattcag gtttcgctgg tcttccagac
1321 tttacagcag atgcgtgata aaacgccgct gtcattaaat acgccgcccg gagaggtgaa
1381 actgaccctg gcaggatgtg aagagcgaaa tgcgcagggc atgtgttcgt tggcaggttt
1441 tacgcaaatc gtgaatgaag cacgcatacc ggcgtgcagt ttgtaatgca taaaaaagag
1501 cattcagtta cctgaatgct ctgaggctga tgacaaacga agaactgtct aatgcgtaga
1561 ccggaaaagg cgttcacgcc gcatccggcc actttcagtt ttcctctttc tcggagtaac
1621 tataaccgta atagttatag ccgtaactgt aagcggtgct ggcgcgttta atcacaccat
1681 tgaggatagc gcctttaata ttgacgcctg cctgttccag acgctgcatt gacaaactca
1741 cctctttggc ggtgttcaag ccaaaacgcg caaccagcag gctggtgcca acagaacgcc
1801 ccacgaccgc ggcatcactc accgccagca tcggcggcgt atcgacaatc accagatcgt
1861 aatggtcgtt cgcccattcc agtaattgac gcatccgatc g

FIGURE 7B

1 taaggagcag aaacaatgtg gtatttactt tggttcgtcg gcattttgtt gatgtgttcg
61 ctctccaccc ttgtgttggt atggctggac ccgcgtctga aaagttaacg aacgtaggcc
121 tgatgcggcg cattagcatc gcatcaggca atcaataatg tcagatatga aaagcggaaa
181 catatcgatg aaagcgatct taatcccatt tttatctctt ctgattccgt taacccccgca
241 atctgcattc gctcagagtg agccggagct gaagctggaa agtgtggtga ttgtcagtcg
301 tcatggtgtg cgtgctccaa ccaaggccac gcaactgatg caggatgtca ccccagacgc
361 atggccaacc tggccggtaa aactgggttg gctgacaccg cgnggtggtg agctaatcgc
421 ctatctcgga cattaccaac gccagcgtct ggtagccgac ggattgctgg cgaaaaaggg
481 ctgcccgcag tctggtcagg tcgcgattat tgctgatgtc gacgagcgta cccgtaaaac
541 aggcgaagcc ttcgccgccg ggctggcacc tgactgtgca ataaccgtac atacccaggc
601 agatacgtcc agtcccgatc cgttatttaa tcctctaaaa actggcgttt gccaactgga
661 taacgcgaac gtgactgacg cgatcctcag cagggcagga gggtcaattg ctgactttac
721 cgggcatcgg caaacggcgt ttcgcgaact ggaacgggtg cttaattttc cgcaatcaaa
781 cttgtgcctt aaacgtgaga aacaggacga aagctgttca ttaacgcagg cattaccatc
841 ggaactcaag gtgagcgccg acaatgtctc attaaccggt gcggtaagcc tcgcatcaat
901 gctgacggag atatttctcc tgcaacaagc acagggaatg ccggagccgg ggtggggaag
961 gatcaccgat tcacaccagt ggaacacctt gctaagtttg cataacgcgc aattttattt
1021 gctacaacgc acgccagagg ttgcccgcag ccgcgccacc ccgttattag atttgatcaa
1081 gacagcgttg acgccccatc caccgcaaaa acaggcgtat ggtgtgacat tacccacttc
1141 agtgctgttt atcgccggac acgatactaa tctggcaaat ctcggcggcg cactggagct
1201 caactggacg cttcccggtc agccggataa cacgccgcca ggtggtgaac tggtgtttga
1261 acgctggcgt cggctaagcg ataacagcca gtggattcag gtttcgctgg tcttccagac
1321 tttacagcag atgcgtgata aaacgccgct gtcattaaat acgccgcccg gagaggtgaa
1381 actgaccctg gcaggatgtg aagagcgaaa tgcgcagggc atgtgttcgt tggcaggttt
1441 tacgcaaatc gtgaatgaag cacgcatacc ggcgtgcagt ttgtaatgca taaaaaagag
1501 cattcagtta cctgaatgct ctgaggctga tgacaaacga agaactgtct aatgcgtaga
1561 ccggaaaagg cgttcacgcc gcatccggcc actttcagtt ttcctctttc tcggagtaac
1621 tataaccgta atagttatag ccgtaactgt aagcggtgct ggcgcgttta atcacaccat
1681 tgaggatagc gcctttaata ttgacgcctg cctgttccag acgctgcatt gacaaactca
1741 cctctttggc ggtgttcaag ccaaaacgcg caaccagcag gctggtgcca acagaacgcc
1801 ccacgaccgc ggcatcactc accgccagca tcggcggcgt atcgacaatc accagatcgt
1861 aatggtcgtt cgcccattcc agtaattgac gcatccgatc g

FIGURE 8

Amino acid sequence for E. coli appA (wild type) (SEQ ID NO:8)

MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAPTKATQLMQDVTPDAWP
TWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQSGQVAIIADVDERTRK
TGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA
NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPSE
LKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQFY
LLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGGAL
ELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNTPP
GEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL

Bold-Underlined amino acid residues are shown below in the modified appA enzyme (SEQ ID NO:10)

MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAPTKATQLMQDVTPDAWP
TWPVKLGELTPRGGELIAYLGHYWRQRLVADGLLPKCGCPQSGQVAIIADVDERTRK
TGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA
NVTDAILEAGGSIADFTGHYQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPSEL
KVSADCVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQFDL
QRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGGALEL
NWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNTPPGE
VKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL